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(54) **Title:** FATTY ACID ELONGATION GENES AND USES THEREOF

(57) **Abstract:** Human and mouse genes involved in fatty acid elongation and related to *CIG30*. Nucleic acids, polypeptides, oligonucleotide probes and primers, methods of diagnosis or prognosis, and other methods relating to and based on the genes, and generation of animal models for deficiencies in one or more of these genes, useful particularly in study of various disorders, especially skin or eye disorders, and in assay methods for obtaining agents of therapeutic potential in such disorders.

FATTY ACID ELONGATION GENES AND USES THEREOF

The present invention relates to nucleic acids, polypeptides, oligonucleotide probes and primers, methods of diagnosis or prognosis, and other methods relating to and based on the identification of several genes involved in fatty acid elongation, and generation of animal models for deficiencies in one or more of these genes, useful particularly in study of various disorders, especially skin or eye disorders, and in assay methods for obtaining agents of therapeutic potential in such disorders.

More particularly, the present invention is based on cloning and characterisation of genes which the present inventors have termed *Ssc1* and *Ssc2*. Mouse and human homologues are provided. Further work providing basis for aspects of the present invention involved characterisation and use of these genes and the previously cloned related gene *Cig30*.

A number of human disorders are known to be related to abnormal levels of very long chain fatty acids (VLCFA) such as peroxisomal disorders (nine out of fifteen), myelin deficiency. There are also several pathological cases, such as e.g. tumor invasion, which are related to a disturbed

metabolism of sphingolipid and ceramides, in which VLCFA are part of. A rational analysis of VLCFA and sphingolipid metabolism has not been possible since the condensing enzymes which are responsible for the elongation process have not been isolated.

Fatty acyl chains account for more than half of the mass of most major phospholipids and are primarily responsible for the apolar nature of the membrane bilayer. Depending on their chain length and degree of unsaturation, they contribute to fluidity and other physical and chemical properties of the membrane. De novo synthesis of fatty acids by the soluble, cytosolic enzymes of the acetyl-CoA carboxylase and fatty acid synthase complexes produces mainly palmitate (16-carbons), with minor amounts of stearate (18-carbons). Quantitatively, these chain lengths are major components of many membrane lipids and qualitatively appear to be related to the optimum width of the membrane lipid bilayer. On the other hand, in membranes many major acyl chains are longer than 16-carbons. For example, in the myelin surrounding axonal processes of neuronal cells, fatty acyl chains of 18-carbons or greater make up more than 60% of the total, and in sphingolipids in particular, acyl chains of 24-carbons are prominent. In liver, brain and other tissues there are two primary systems

for elongation, one in the endoplasmic reticulum, and the other in mitochondria.

Microsomal chain elongation appears to be the major source of acyl chains greater than 16-carbons during growth and maturation, when required long chain acids may not be supplied in the diet. For example, 18- to 24-carbons are required for normal brain myelination, regardless of dietary fluctuations during development.

Furthermore, as major membrane components, acyl chains influence a variety of membrane functions, such as ion channels and transport, endocytosis and exocytosis, and the activities of membrane-associated enzymes. These processes are proposed to occur through dynamic, so called, "rafts" which are made up specific sphingolipids and cholesterol within the plasma membrane. Rafts, together with the structural proteins caveolins, are the major structure of caveolae which are vesicular invaginations of the plasma membrane. In these caveolae many classes of signalling molecules are assembled to generate functional signal transducers, including e.g. G-proteins, Ras- and Src family tyrosine kinases, Protein Kinase C isoforms, EGF receptors, Nitric Oxide Synthase, etc, within the plasma membrane.

The regulation of the eukaryotic stress response involves the operation of multiple and poorly defined regulatory and signal transduction pathways that sense the degree and type of stress and reprogram it to a cell response. Multiple lines of evidence implicate sphingolipids, ceramides and sphingosines as key regulators of the eukaryotic stress response, e.g. ceramides formed after activation of sphingomyelinase (by TNFa and IL-1) decrease cell division and induce apoptosis which on the other hand can be counteracted by sphingosine-phosphate which stimulate cell growth and inhibit apoptosis.

The inventors isolated a full-length cDNA of a previously uncharacterised gene now termed *Cig30* (Tvrdik et al., J. Biol. Chem. (1997) 272: 31738-31746).

In the public databases, there are no mammalian proteins resembling *Cig30*, but three yeast proteins (JO343, FEN1 and SUR4) are significantly homologous to *Cig30*. Interestingly, the yeast genes have recently been suggested to function as membrane-bound fatty acid elongases and designated ELO1(JO343), ELO2(FEN1) and ELO3(SUR4) where ELO1 elongates fatty acids specifically up to 16-carbons and ELO2 and ELO3 specifically elongate up to very long chain fatty acids, 24-carbons and 26-carbons respectively (C. Oh, D.A. Toke, S.

Mandala and C.E. Martin, J. Biol. Chem. 272; 17376-84, 1997) mutations in the corresponding genes give rise to disorders in several metabolic pathways as well as disorganised cytoskeleton, impaired regulation of cellgrowth and budding deficiency (L. Desfarge et al., Yeast 9, 267-77, 1993; E. Revardel et al., Biochem. Biophys. Acta 1263, 261-265, 1995; S. Silve et al., Mol. Cell Bio., 16, 2719-27, 1996).

Disruption of either *ELO2* or *ELO3* is associated with lower levels of sphingolipids as a result of reduced ceramide synthesis (Oh et al., (1997) *J. Biol. Chem.* 272, 17376-17384).

This can provide a rational explanation to all the pleiotropic phenotypes seen in *SUR4* and *FEN1* mutations. The three yeast members have been characterised by several independent groups and therefor been given different names. *SUR4* was originally selected as a suppressor of the phenotypes of the rvs-strains (reduced viability upon starvation). Mutants for *RVS161* and *RSV167* are identified by heterologous cell morphology upon starvation followed by deficient bud localisation and cell lysis. Further, the *SUR4* mutant cells show abnormal morphologies in stationary phase as well as a decrease in phospholipids (Desfarges et al.(1993) *Yeast* 9, 267-277). It was also observed that the same mutant confers resistance when

exposed to the immunosuppressor SR31747 in yeast cells which inhibits sterol isomerase in the synthesis of ergosterol (Silve et al., (1996) *Mol. Cell. Biol.* 16. 2719-2727). The SUR4 mutation also show a decreased level of plasma membrane H+-ATPase and several genes related to glucose metabolism (Garcia-Arranz et al., (1994) *J. Biol. Chem.* 269, 18076-18082).

The *FEN1* mutation was initially selected due to its ability to confer resistance to inhibition of sterol synthesis, particular by fenpropimorph (Ladeveze et al., (1993) *Lipids* 28, 907-912). The same resistance towards fenpropimorph is also seen by *SUR4*. Mutation of *FEN1* exhibit similar phenotypes like the *SUR4* mutations, i.e. the ability to suppress *RVS161* and *RSV167*, increased generation time, bud localisation defect and resistance to SR31747. Also, yeast cells deficient in *FEN1* show a decrease in 1,3-β-glucan synthesis which catalyses the polymerisation of glucose necessary for the cell wall (El-Sherbeini and Clemas., (1995) *J. Bacteriol.* 177, 3227-3234). Simultaneous disruption of *SUR4* and *FEN1* produces a lethal phenotype, which indicates that their encoded proteins have related functions (Silve et al., (1996) *Mol. Cell. Biol.* 16, 2719-2727). The third mutation, in the gene called J0343, shows no different

phenotype from wild-type cells and had recently no detectable function (Revardel et al., (1995) *Biochim. Biophys. Acta* 1263, 261-265).

The inventors have now cloned two genes, *Ssc1* and *Ssc2* (human and mouse). *Ssc1* mRNA seems to be ubiquitous present in all tissues, while *Ssc2* mRNA has only been detected in testis and liver.

The inventors subcloned *Cig30*, as well as *Ssc1* and *Ssc2*, into a yeast expression vector, and transformed the genes into sur4 and fen1 yeast mutants. The transformants were tested for wildtype phenotype. The data show that complementation clearly occurs and indicates that *Cig30* and *Ssc2* are functional equivalents to ELO2, whereas *Ssc1* is equivalent to ELO3.

This provides indication that *Cig30, Ssc1* and *Ssc2* are involved in the formation of very long fatty acids (VLCFA).

As VLCFA are mainly precursors for ceramide and sphingolipid synthesis, the inventors measured the levels of specific ceramides and sphingolipids in the transformants by thin-layer-chromatography after incubation with radioactive serine

as precursor. The data confirm that *Cig30* and *Ssc1* can restore the levels of specific ceramides and sphingolipids in the corresponding yeast mutants.

Further work described below has established deficiency in *Ssc1* gene expression in myelin deficient mice. Furthermore, *Cig30* knockout mice have surprisingly been found to provide phenotypes representing a good model for skin disease, the gene apparently being involved in skin development. Such mice and equivalent *Ssc1* and *Ssc2* knockout mice provide for assays for identifying and obtaining agents which may be used for treatment of skin disorders, disorders of hair growth, fertility (especially *Ssc2*), multiple sclerosis (MS - especially *Ssc1*), obesity, cachexia, and thermoregulation (especially *Cig30*). Additionally, the mice show eye problems within the first 3-5 weeks after birth. This provides for testing of substances for treatment of eye disorders or other problems (e.g. when contact lenses are used).

Further assays are provided by the invention, employing the *Ssc1* and *Ssc2* polynucleotides and encoded polypeptides in identifying and obtaining agents of therapeutic potential in peroxisomal disorders, cancers, and other disorders as discussed herein and will be apparent to those skilled in the

art in view of the present disclosure.

The peroxisomal disorders display frequently the phenomena of several genetic defects/mechanisms, leading to multiple phenotypic effects such as multiple congenital anomalies and severe neurological deficits. All these are associated with defects in peroxisomal beta oxidation.

Pathologically high levels of very long chain fatty acids are often found in plasma of patients with peroxisomal disorders, such as Zellweger syndrome, neonatal adrenoleukodystrophy (NALD), infantile Resum disease (IRD) and the rhizomelic chondrodysplasia puncata (RCDP) because of the impaired oxidation of very long chain fatty acids by peroxisomes.

Saturated VLCFA accumulate in ALD, appear to disrupt membrane structure, and may play a role in the pathogenesis of bain inflammatory response. The glyceryl trierucate oil, Lorenzo's oil, combined with a reduced dietary fat intake, normalises levels of VLCFA in plasma within 4 wk. The oil is thought to act by reducing endogenous synthesis of saturated VLCFA by microsomal elongation system (see review H. Moser and A. Moser, 1996, Lipids 31, S141-144.

Furthermore, in the adrenoleukodystrophy case, dietary restriction of very long chain fatty acids has been tried as therapy but with very little improvement of clinical symptoms. In addition to the aberrant degradation activity of VLCFA in this disease, there has been reported a significant increased synthesis of VLCFA in ALD fibroblasts which suggests that this is of importance for the accumulation of the VLCFA in this disease as well (Tsuji et al., J. Biochem. 96, 1241-1247, 1984).

Gangliosides and sphingolipids modulate transmembrane signalling essential for tumour cell growth, invasion and metastasis. The transducer molecules susceptible to gangliosides and sphingolipids include integrin receptors, tyrosine kinase-linked growth factor receptors, protein kinase C, and G-protein-linked receptor affecting protein kinase A. Some glycosphingolipids, ceramides and sphingosine induce tumour cell differentiation and subsequently apoptosis.

Ceramide accumulation has been reported in Rous sarcoma transformed fibroblasts. In addition, natural killer T (NKT) lymphocytes express an invariant T cell receptor which uses glycosylceramides with very long chain fatty acid (C26) as ligand which exist in restricted mammalian tissues or

expressed on cells after activation or during malignancy (T. Kawano et al., 1997, Science 278, 1626-1629).

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows mouse *Ssc1* cDNA sequence, including coding sequence.

Figure 2 shows mouse SSC1 amino acid sequence.

Figure 3 shows human *Ssc1* cDNA sequence, including coding sequence.

Figure 4 shows human SSC1 amino acid sequence.

Figure 5 shows mouse *Ssc2* cDNA sequence, including coding sequence.

Figure 6 shows mouse SSC2 amino acid sequence.

Figure 7 shows an alignment of SSC1, SSC2 and CIG30 protein and yeast protein sequences.

Figure 8 shows a schematic overview of genomic *Cig30*,

identified as Plaque A in plaque hybridisation as described below. The insert of approximately 14kb is in the Lambda FIXII vector. The boxes indicate exon 1-4 of *Cig30*. Below are the two plasmids shown which correspond to the two arms in the gene targeting vector. The dotted lines show to which part in Plaque A the plasmid corresponds and which restriction enzymes were used to digest Plaque A. The lower plasmid, large right arm 5.30 kb, was initially used to create the gene targeting vector. The inserts are cloned into the Bluescript plasmid, pBS.

Figure 9 illustrates sequence replacement of *Cig30* by the corresponding gen targeting construct.

Figure 9A shows the knock-out vector for *Cig30* (top) replaces the transcription start and exon 1 and 2 of the genomic DNA (below) upon homologous recombination with the *neo*^{r} gene (crosshatched box). The *neo*^{r} replaces exon 1 and 2 (bend part) in the endogenous sequence. The black box indicates the thymidine kinase (*tk*) gene with its corresponding PYF enhancer. The light grey areas indicate the left (LA) and right (RA) arm which pair with a chromosomal copy of *Cig30* (dotted lines). Medium grey shows the 5'UTR and the 3'UTR regions and dark grey exon 1 to exon 4. Above the gene targeting construct are the positions of restriction enzymes.

Restriction enzymes within brackets were deleted during the cloning procedure.

Figure 9B shows a schematic model of the final incorporation of the gene targeting constructs into the genome.

Figure 10 illustrates a flow scheme with the pathway used in making knock-out mice.

The Figure 1A of Tvrdik et al., J. Biol. Chem. (1997) 272: 31738-31746 is also specifically incorporated herein by reference as if it appears in full. It shows the nucleotide sequence of mouse *Cig30* cDNA, including coding region, and predicted amino acid sequence of CIG30 protein. Putative membrane-spanning segments are underlined with continuous lines and numbered with Roman numerals. An N-glycosylation consensus site (amino acids 6-9) is underlined with a dashed line. An arbitrary stretch of amino acids in the central part of the polypeptide (amino acids 116-150), which displays the highest degree of homology throughout the family, is marked by a dotted line. The four leucines defining the leucine zipper within transmembrane domain IV are italicized, and the poly(A) signal is marked by a grey box.

Sequence data deposited with the GenBank Data Library under Accession No. AF054504 are also incorporated herein by reference.

According to one aspect of the present invention there is provided a nucleic acid molecule or polynucleotide which has a nucleotide sequence encoding a polypeptide which includes an amino acid sequence selected from the group consisting of:

(1) the mouse Ssc1 amino acid sequence shown herein;

(2) the mouse Ssc2 amino acid sequence shown herein;

(3) the human Ssc1 amino acid sequence shown herein;

(4) a fragment or active portion of any of (1), (2) or (3) as explained further below;

(5) a variant or derivative of any of (1), (2) or (3) as explained further below.

The coding sequence may be the relevant one shown herein, or it may be a mutant, variant, derivative or allele of the sequence shown. The sequence may differ from that shown by a change which is one or more of addition, insertion, deletion and substitution of one or more nucleotides of the sequence shown. Changes to a nucleotide sequence may result in an amino acid change at the protein level, or not, as determined by the genetic code.

Thus, nucleic acid according to the present invention may include a sequence different from a sequence specifically shown herein, yet encode a polypeptide with the same amino acid sequence.

On the other hand the encoded polypeptide may comprise an amino acid sequence which differs by one or more amino acid residues from the amino acid sequence shown herein. Nucleic acid encoding a polypeptide which is an amino acid sequence mutant, variant or derivative of the sequence shown is further provided by the present invention. Such polypeptides are discussed below. Nucleic acid encoding such a polypeptide may show at the nucleotide sequence and/or encoded amino acid level greater than about 60% homology with the relevant coding or encoded sequence shown herein, greater than about 70% homology, greater than about 80% homology, greater than about 90% homology or greater than about 95% homology. For amino acid "homology", this may be understood to be similarity (according to the established principles of amino acid similarity, e.g. as determined using the algorithm GAP (Genetics Computer Group, Madison, WI) or identity. GAP uses the Needleman and Wunsch algorithm to align two complete sequences that maximizes the number of matches and minimizes the number of gaps. Generally, the default parameters are

used, with a gap creation penalty = 12 and gap extension penalty = 4. Use of GAP may be preferred but other algorithms may be used, e.g. BLAST (which uses the method of Altschul *et al.* (1990) *J. Mol. Biol.* **215**: 405-410), FASTA (which uses the method of Pearson and Lipman (1988) *PNAS USA* **85**: 2444-2448), or the Smith-Waterman algorithm (Smith and Waterman (1981) *J. Mol Biol.* ***147***: 195-197), generally employing default parameters. Use of either of the terms "homology" and "homologous" herein does not imply any necessary evolutionary relationship between compared sequences, in keeping for example with standard use of terms such as "homologous recombination" which merely requires that two nucleotide sequences are sufficiently similar to recombine under the appropriate conditions. Further discussion of polypeptides according to the present invention, which may be encoded by nucleic acid according to the present invention, is found below.

The present invention extends to nucleic acid that hybridizes with any one or more of the specific sequences disclosed herein under stringent conditions. Suitable conditions include, e.g. for detection of sequences that are about 80-90% identical suitable conditions include hybridization overnight at 42°C in 0.25M Na_2HPO_4, pH 7.2, 6.5% SDS, 10% dextran sulfate

and a final wash at 55°C in 0.1X SSC, 0.1% SDS. For detection of sequences that are greater than about 90% identical, suitable conditions include hybridization overnight at 65°C in 0.25M Na_2HPO_4, pH 7.2, 6.5% SDS, 10% dextran sulfate and a final wash at 60°C in 0.1X SSC, 0.1% SDS.

Thus, for example the present invention further extends to a polynucleotide which hybridizes under stringent conditions with a *Cig30*, *Ssc1* and/or *Ssc2* sequence as disclosed herein. An example of an embodiment of a polynucleotide encoding a *Ssc1* human homologue of a mouse sequence provided herein is shown herein.

The human and mouse SSC1 have 92.3% similarity as calculated using BLAST with standard algorithm parameters W, T and X and the BLOSUM62 matrix (Altschul, et al., *supra*), 92.5% identity using BESTFIT (see below) over the full length 279 amino acids. Suitably stringent conditions for selective hybridisation between the mouse and human homologues may be provided in accordance with the explanation and formula as follows.

Preliminary experiments may be performed by hybridising under low stringency conditions. For probing, preferred conditions

are those which are stringent enough for there to be a simple pattern with a small number of hybridisations identified as positive which can be investigated further.

For example, hybridizations may be performed, according to the method of Sambrook et al. (below) using a hybridization solution comprising: 5X SSC (wherein `SSC' = 0.15 M sodium chloride; 0.15 M sodium citrate; pH 7), 5X Denhardt's reagent, 0.5-1.0% SDS, 100 μg/ml denatured, fragmented salmon sperm DNA, 0.05% sodium pyrophosphate and up to 50% formamide. Hybridization is carried out at 37-42°C for at least six hours. Following hybridization, filters are washed as follows: (1) 5 minutes at room temperature in 2X SSC and 1% SDS; (2) 15 minutes at room temperature in 2X SSC and 0.1% SDS; (3) 30 minutes - 1 hour at 37°C in 1X SSC and 1% SDS; (4) 2 hours at 42-65°C in 1X SSC and 1% SDS, changing the solution every 30 minutes.

One common formula for calculating the stringency conditions required to achieve hybridization between nucleic acid molecules of a specified sequence homology is (Sambrook et al., 1989): $T_m = 81.5°C + 16.6 \mathrm{Log}\,[Na+] + 0.41\,(\%\ G+C) - 0.63\,(\%\ \mathrm{formamide}) - 600/\#bp$ in duplex.

As an illustration of the above formula, using [Na+] = [0.368] and 50-% formamide, with GC content of 42% and an average probe size of 200 bases, the T_m is 57°C. The T_m of a DNA duplex decreases by 1 - 1.5°C with every 1% decrease in homology. Thus, targets with greater than about 75% sequence identity would be observed using a hybridization temperature of 42°C. Such a sequence would be considered substantially homologous to the nucleic acid sequence of the present invention.

It is well known in the art to increase stringency of hybridisation gradually until only a few positive clones remain. Other suitable conditions include, e.g. for detection of sequences that are about 80-90% identical, hybridization overnight at 42°C in 0.25M Na_2HPO_4, pH 7.2, 6.5% SDS, 10% dextran sulfate and a final wash at 55°C in 0.1X SSC, 0.1% SDS. For detection of sequences that are greater than about 90% identical, suitable conditions include hybridization overnight at 65°C in 0.25M Na_2HPO_4, pH 7.2, 6.5% SDS, 10% dextran sulfate and a final wash at 60°C in 0.1X SSC, 0.1% SDS.

Generally, nucleic acid according to the present invention is provided as an isolate, in isolated and/or purified form, or

free or substantially free of material with which it is naturally associated, such as free or substantially free of nucleic acid flanking the gene in the human genome, except possibly one or more regulatory sequence(s) for expression. Nucleic acid may be wholly or partially synthetic and may include genomic DNA, cDNA or RNA. The coding sequence shown herein is a DNA sequence. Where nucleic acid according to the invention includes RNA, reference to the sequence shown should be construed as encompassing reference to the RNA equivalent, with U substituted for T.

Nucleic acid may be provided as part of a replicable vector, and also provided by the present invention are a vector including nucleic acid as set out above, particularly any expression vector from which the encoded polypeptide can be expressed under appropriate conditions, and a host cell containing any such vector or nucleic acid. An expression vector in this context is a nucleic acid molecule including nucleic acid encoding a polypeptide of interest and appropriate regulatory sequences for expression of the polypeptide, in an *in vitro* expression system, e.g. reticulocyte lysate, or *in vivo*, e.g. in eukaryotic cells such as COS or CHO cells or in prokaryotic cells such as *E. coli*. This is discussed further below.

The nucleic acid sequence provided in accordance with the present invention is useful for identifying nucleic acid of interest (and which may be according to the present invention) in a test sample. The present invention provides a method of obtaining nucleic acid of interest, the method including hybridisation of a probe having the an *Ssc1* or *Ssc2* sequence shown herein, or a complementary sequence, to target nucleic acid. Hybridisation is generally followed by identification of successful hybridisation and isolation of nucleic acid which has hybridised to the probe, which may involve one or more steps of PCR. It will not usually be necessary to use a probe with the complete sequence shown in any of these figures. Shorter fragments may be used, e.g. fragments of about 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190 or 200 nucleotides.

Nucleic acid according to the present invention is obtainable using one or more oligonucleotide probes or primers designed to hybridise with one or more fragments of the nucleic acid sequence shown in any of the figures, particularly fragments of relatively rare sequence, based on codon usage or statistical analysis. A primer designed to hybridise with a fragment of the nucleic acid sequence shown in any of the figures may be used in conjunction with one or more

oligonucleotides designed to hybridise to a sequence in a cloning vector within which target nucleic acid has been cloned, or in so-called "RACE" (rapid amplification of cDNA ends) in which cDNA's in a library are ligated to an oligonucleotide linker and PCR is performed using a primer which hybridises with a sequence shown and a primer which hybridises to the oligonucleotide linker.

Such oligonucleotide probes or primers, as well as the full-length sequence (and mutants, alleles, variants and derivatives) are also useful in screening a test sample containing nucleic acid for the presence of alleles, mutants and variants, with diagnostic and/or prognostic implications as discussed in more detail below.

Nucleic acid isolated and/or purified from one or more cells (e.g. human) or a nucleic acid library derived from nucleic acid isolated and/or purified from cells (e.g. a cDNA library derived from mRNA isolated from the cells), may be probed under conditions for selective hybridisation and/or subjected to a specific nucleic acid amplification reaction such as the polymerase chain reaction (PCR) (reviewed for instance in "PCR protocols; A Guide to Methods and Applications", Eds. Innis et al, 1990, Academic Press, New York, Mullis et al, Cold

Spring Harbor Symp. Quant. Biol., 51:263, (1987), Ehrlich (ed), PCR technology, Stockton Press, NY, 1989, and Ehrlich et al, Science, 252:1643-1650, (1991)). PCR comprises steps of denaturation of template nucleic acid (if double-stranded), annealing of primer to target, and polymerisation. The nucleic acid probed or used as template in the amplification reaction may be genomic DNA, cDNA or RNA. Other specific nucleic acid amplification techniques include strand displacement activation, the QB replicase system, the repair chain reaction, the ligase chain reaction and ligation activated transcription. For convenience, and because it is generally preferred, the term PCR is used herein in contexts where other nucleic acid amplification techniques may be applied by those skilled in the art. Unless the context requires otherwise, reference to PCR should be taken to cover use of any suitable nucleic amplification reaction available in the art.

In the context of cloning, it may be necessary for one or more gene fragments to be ligated to generate a full-length coding sequence. Also, where a full-length encoding nucleic acid molecule has not been obtained, a smaller molecule representing part of the full molecule, may be used to obtain full-length clones. Inserts may be prepared from partial cDNA

clones and used to screen cDNA libraries. The full-length clones isolated may be subcloned into expression vectors and activity assayed by transfection into suitable host cells, e.g. with a reporter plasmid.

A method may include hybridisation of one or more (e.g. two) probes or primers to target nucleic acid. Where the nucleic acid is double-stranded DNA, hybridisation will generally be preceded by denaturation to produce single-stranded DNA. The hybridisation may be as part of a PCR procedure, or as part of a probing procedure not involving PCR. An example procedure would be a combination of PCR and low stringency hybridisation. A screening procedure, chosen from the many available to those skilled in the art, is used to identify successful hybridisation events and isolated hybridised nucleic acid.

Binding of a probe to target nucleic acid (e.g. DNA) may be measured using any of a variety of techniques at the disposal of those skilled in the art. For instance, probes may be radioactively, fluorescently or enzymatically labelled. Other methods not employing labelling of probe include examination of restriction fragment length polymorphisms, amplification using PCR, RN'ase cleavage and allele specific oligonucleotide

probing. Probing may employ the standard Southern blotting technique. For instance DNA may be extracted from cells and digested with different restriction enzymes. Restriction fragments may then be separated by electrophoresis on an agarose gel, before denaturation and transfer to a nitrocellulose filter. Labelled probe may be hybridised to the DNA fragments on the filter and binding determined. DNA for probing may be prepared from RNA preparations from cells.

Preliminary experiments may be performed by hybridising under low stringency conditions various probes to Southern blots of DNA digested with restriction enzymes. Suitable conditions would be achieved when a large number of hybridising fragments were obtained while the background hybridisation was low. Using these conditions nucleic acid libraries, e.g. cDNA libraries representative of expressed sequences, may be searched. Those skilled in the art are well able to employ suitable conditions of the desired stringency for selective hybridisation, taking into account factors such as oligonucleotide length and base composition, temperature and so on. On the basis of amino acid sequence information, oligonucleotide probes or primers may be designed, taking into account the degeneracy of the genetic code, and, where appropriate, codon usage of the organism from the candidate

nucleic acid is derived. An oligonucleotide for use in nucleic acid amplification may have about 10 or fewer codons (e.g. 6, 7 or 8), i.e. be about 30 or fewer nucleotides in length (e.g. 18, 21 or 24, 10-15, 10-20, 15-20 or 20-30). Generally specific primers are upwards of 14 nucleotides in length, but need not be than 18-20. Those skilled in the art are well versed in the design of primers for use processes such as PCR. Various techniques for synthesizing oligonucleotide primers are well known in the art, including phosphotriester and phosphodiester synthesis methods.

A further aspect of the present invention provides an oligonucleotide or polynucleotide fragment of the nucleotide sequence shown in any of the figures herein providing nucleic acid according to the present invention, or a complementary sequence, in particular for use in a method of obtaining and/or screening nucleic acid. Some preferred oligonucleotides have a sequence shown herein, or a sequence which differs from any of the sequences shown by addition, substitution, insertion or deletion of one or more nucleotides, but preferably without abolition of ability to hybridise selectively with nucleic acid in accordance with the present invention, that is wherein the degree of similarity of the oligonucleotide or polynucleotide with one of the

sequences given is sufficiently high.

In some preferred embodiments, oligonucleotides according to the present invention that are fragments of any of the sequences shown, or any allele associated with a disorder or other disease susceptibility, may be or consist of at least about 10 nucleotides in length, more preferably at least about 15 nucleotides in length, more preferably at least about 20 nucleotides in length, at least about 30 nucleotides in length, more preferably at least about 40 nucleotides in length, more preferably at least about 50 nucleotides in length. Fragments may be 10-20 nucleotides, 10-30, 20-30, 30-40, 30-50, 40-50, 50-60, 50-100, 50-150 or 100-150 nucleotides in length. Such fragments themselves individually represent aspects of the present invention. Fragments and other oligonucleotides may be used as primers or probes as discussed but may also be generated (e.g. by PCR) in methods concerned with determining the presence in a test sample of a sequence indicative of a disorder or disease susceptibility.

Methods may involve use of nucleic acid in diagnostic and/or prognostic contexts, for instance in determining susceptibility to a disease, and other methods are concerned with determining the presence of sequences indicative of a

defect in VLCFA biosynthesis or other disease susceptibility.

Further embodiments of oligonucleotides according to the present invention are anti-sense oligonucleotide sequences based on the nucleic acid sequences described herein. Anti-sense oligonucleotides may be designed to hybridise to the complementary sequence of nucleic acid, pre-mRNA or mature mRNA, interfering with the production of polypeptide encoded by a given DNA sequence (e.g. either native polypeptide or a mutant form thereof), so that its expression is reduce or prevented altogether. Anti-sense techniques may be used to target a coding sequence, a control sequence of a gene, e.g. in the 5' flanking sequence, whereby the antisense oligonucleotides can interfere with control sequences. Anti-sense oligonucleotides may be DNA or RNA and may be of around 14-23 nucleotides, particularly around 15-18 nucleotides, in length. Fragments of lengths identified above may also be employed (in the antisense orientation.) The construction of antisense sequences and their use is described in Peyman and Ulman, Chemical Reviews, 90:543-584, (1990), and Crooke, Ann. Rev. Pharmacol. Toxicol., 32:329-376, (1992).

Nucleic acid according to the present invention may be used in methods of gene therapy, for instance in treatment of

individuals with the aim of preventing or curing (wholly or partially) a disorder or defect in VLCFA biosynthesis or other disease. This may ease one or more symptoms of the disease. This is discussed below.

Nucleic acid according to the present invention, such as a full-length coding sequence or oligonucleotide probe or primer, may be provided as part of a kit, e.g. in a suitable container such as a vial in which the contents are protected from the external environment. The kit may include instructions for use of the nucleic acid, e.g. in PCR and/or a method for determining the presence of nucleic acid of interest in a test sample. A kit wherein the nucleic acid is intended for use in PCR may include one or more other reagents required for the reaction, such as polymerase, nucleosides, buffer solution etc. The nucleic acid may be labelled. A kit for use in determining the presence or absence of nucleic acid of interest may include one or more articles and/or reagents for performance of the method, such as means for providing the test sample itself, e.g. a swab for removing cells from the buccal cavity or a syringe for removing a blood sample (such components generally being sterile).

A further aspect of the present invention provides a

polypeptide which has the amino acid sequence of a Ssc1 or Ssc2 polypeptide shown herein, which may be in isolated and/or purified form, free or substantially free of material with which it is naturally associated, such as other polypeptides or such as human or mouse polypeptides other than that for which the relevant amino acid sequence is shown herein, or (for example if produced by expression in a prokaryotic cell) lacking in native glycosylation, e.g. unglycosylated.

Polypeptides which are amino acid sequence variants, alleles, derivatives or mutants are also provided by the present invention. A polypeptide which is a variant, allele, derivative or mutant may have an amino acid sequence which differs from that given in a figure herein by one or more of addition, substitution, deletion and insertion of one or more amino acids. Preferred such polypeptides have whichever is the relevant function out of CIG30, SSC1 and SSC2 function, that is to say have one or more of the following properties: immunological cross-reactivity with an antibody reactive with the relevant polypeptide for which the sequence is given herein; sharing an epitope with the relevant polypeptide for which the amino acid sequence is shown herein (as determined for example by immunological cross-reactivity between the two polypeptides); a biological activity which is inhibited by an

antibody raised against the polypeptide whose sequence is shown in a figure herein; ability to complement ELO1, ELO2 and/or ELO3 mutations in yeast; enzymatic activity in common with CIG 30, SSC1 and/or SSC2. Alteration of sequence may change the nature and/or level of activity and/or stability of the relevant protein.

A polypeptide which is an amino acid sequence variant, allele, derivative or mutant of the amino acid sequence shown in a figure herein may comprise an amino acid sequence which shares greater than about 35% sequence identity with the sequence shown, greater than about 40%, greater than about 50%, greater than about 60%, greater than about 70%, greater than about 80%, greater than about 90% or greater than about 95%. The sequence may share greater than about 60% similarity, greater than about 70% similarity, greater than about 80% similarity or greater than about 90% similarity with the amino acid sequence shown in the relevant figure. Amino acid similarity is generally defined with reference to the algorithm GAP (Genetics Computer Group, Madison, WI) as noted above, or the TBLASTN program, of Altschul et al. (1990) J. Mol. Biol. 215: 403-10. Similarity allows for "conservative variation", i.e. substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or

the substitution of one polar residue for another, such as arginine for lysine, glutamic for aspartic acid, or glutamine for asparagine. Particular amino acid sequence variants may differ from that shown in a figure herein by insertion, addition, substitution or deletion of 1 amino acid, 2, 3, 4, 5-10, 10-20 20-30, 30-50, 50-100, 100-150, or more than 150 amino acids.

Sequence comparison may be made over the full-length of the relevant sequence shown herein, or may more preferably be over a contiguous sequence of about or greater than about 20, 25, 30, 33, 40, 50, 67, 133, 167, 200, 233, 267 or more amino acids or nucleotide triplets, compared with the relevant amino acid sequence or nucleotide sequence as the case may be.

The present invention also includes peptides which include or consist of fragments of a polypeptide of the invention.

The skilled person can use the techniques described herein and others well known in the art to produce large amounts of peptides, for instance by expression from encoding nucleic acid.

Peptides can also be generated wholly or partly by chemical

synthesis. The compounds of the present invention can be readily prepared according to well-established, standard liquid or, preferably, solid-phase peptide synthesis methods, general descriptions of which are broadly available (see, for example, in J.M. Stewart and J.D. Young, Solid Phase Peptide Synthesis, 2nd edition, Pierce Chemical Company, Rockford, Illinois (1984), in M. Bodanzsky and A. Bodanzsky, The Practice of Peptide Synthesis, Springer Verlag, New York (1984); and Applied Biosystems 430A Users Manual, ABI Inc., Foster City, California), or they may be prepared in solution, by the liquid phase method or by any combination of solid-phase, liquid phase and solution chemistry, e.g. by first completing the respective peptide portion and then, if desired and appropriate, after removal of any protecting groups being present, by introduction of the residue X by reaction of the respective carbonic or sulfonic acid or a reactive derivative thereof.

The present invention also includes active portions, fragments, derivatives and functional mimetics of the polypeptides of the invention. An "active portion" of a polypeptide means a peptide which is less than said full length polypeptide, but which retains a biological activity, such as ability to complement ELO1, ELO2 and/or ELO3 mutations

in *S. cerevisiae*. Such an active fragment may be included as part of a fusion protein

A "fragment" of a polypeptide generally means a stretch of amino acid residues of at least about five contiguous amino acids, often at least about seven contiguous amino acids, typically at least about nine contiguous amino acids, more preferably at least about 13 contiguous amino acids, and, more preferably, at least about 20 to 30, at least about 30-40, at least about 40-50, at least bout 5-60, at least about 60-70, at least about 70-80, at least about 80-90, at least about 90-100, or more contiguous amino acids. Fragments of the relevant polypeptide sequence may include antigenic determinants or epitopes useful for raising antibodies to a portion of the amino acid sequence. Alanine scans are commonly used to find and refine peptide motifs within polypeptides, this involving the systematic replacement of each residue in turn with the amino acid alanine, followed by an assessment of biological activity.

Preferred fragments of polypeptides according to the present invention include those with sequences which may be used for instance in raising or isolating antibodies, for instance amino acids 116-150 of any of CIG30, SSC1 and SSC2 (mouse or

human). Variant and derivative peptides, peptides which have an amino acid sequence which differs from one of these sequences by way of addition, insertion, deletion or substitution of one or more amino acids are also provided by the present invention, generally with the proviso that the variant or derivative peptide is bound by an antibody or other specific binding member which binds one of the peptides whose sequence is shown. A peptide which is a variant or derivative of one of the shown peptides may compete with the shown peptide for binding to a specific binding member, such as an antibody or antigen-binding fragment thereof.

Where additional amino acids are included in a peptide, these may be heterologous or foreign to the polypeptide of the invention, and the peptide may be about 20, 25, 30 or 35 amino acids in length. A peptide according to this aspect may be included within a larger fusion protein, particularly where the peptide is fused to a heterologous or foreign sequence, such as a polypeptide or protein domain.

A "derivative" of a polypeptide or a fragment thereof may include a polypeptide modified by varying the amino acid sequence of the protein, e.g. by manipulation of the nucleic acid encoding the protein or by altering the protein itself.

Such derivatives of the natural amino acid sequence may involve one or more of insertion, addition, deletion or substitution of one or more amino acids, which may be without fundamentally altering the qualitative nature of biological activity of the wild type polypeptide. Also encompassed within the scope of the present invention are functional mimetics of active fragments of the polypeptides provided (including alleles, mutants, derivatives and variants). The term "functional mimetic" means a substance which may not contain an active portion of the relevant amino acid sequence, and probably is not a peptide at all, but which retains in qualitative terms biological activity of the natural polypeptide. The design and screening of candidate mimetics is described in detail below.

Other fragments of the polypeptides for which sequence information is provided herein are provided as aspects of the present invention, for instance corresponding to functional domains. One such functional domain may be amino acids 126-150 or amino acids 175-196 of the relevant CIG30, SSC1 or SSC2 protein.

A polypeptide according to the present invention may be isolated and/or purified (e.g. using an antibody) for instance

after production by expression from encoding nucleic acid (for which see below). Thus, a polypeptide may be provided free or substantially free from contaminants with which it is naturally associated (if it is a naturally-occurring polypeptide). A polypeptide may be provided free or substantially free of other polypeptides. Polypeptides according to the present invention may be generated wholly or partly by chemical synthesis. The isolated and/or purified polypeptide may be used in formulation of a composition, which may include at least one additional component, for example a pharmaceutical composition including a pharmaceutically acceptable excipient, vehicle or carrier. A composition including a polypeptide according to the invention may be used in prophylactic and/or therapeutic treatment as discussed below.

A polypeptide, peptide, allele, mutant, derivative or variant according to the present invention may be used as an immunogen or otherwise in obtaining specific antibodies. Antibodies are useful in purification and other manipulation of polypeptides and peptides, diagnostic screening and therapeutic contexts. This is discussed further below.

A polypeptide according to the present invention may be used

in screening for molecules which affect or modulate its activity or function. Such molecules may interact with a portion of the polypeptide, and may be useful in a therapeutic (possibly including prophylactic) context.

It is well known that pharmaceutical research leading to the identification of a new drug may involve the screening of very large numbers of candidate substances, both before and even after a lead compound has been found. This is one factor which makes pharmaceutical research very expensive and time-consuming. Means for assisting in the screening process can have considerable commercial importance and utility. Such means for screening for substances potentially useful in treating or preventing a disorder or disease is provided by polypeptides according to the present invention. Substances identified as modulators of the polypeptide represent an advance in the fight against disease since they provide basis for design and investigation of therapeutics for *in vivo* use. Furthermore, they may be useful in any of a number of conditions, including diseases and disorders involving skin development, hair growth, body fat mass (subcutaneous), fertility, thermoregulation, and/or the eye, given the functional indications discussed elsewhere herein. As noted elsewhere, polypeptides, fragments thereof, and nucleic acid

according to the invention may also be useful in combatting any of these diseases and disorders.

In various further aspects the present invention relates to screening and assay methods and means, and substances identified thereby.

Thus, further aspects of the present invention provide the use of a polypeptide or peptide (particularly a fragment of a polypeptide of the invention as disclosed, and/or encoding nucleic acid therefor, in screening or searching for and/or obtaining/identifying a substance, e.g. peptide or chemical compound, which interacts and/or binds with the polypeptide or peptide and/or interferes with its function or activity or that of another substance, e.g. polypeptide or peptide, which interacts and/or binds with the polypeptide or peptide of the invention. For instance, a method according to one aspect of the invention includes providing a polypeptide or peptide of the invention and bringing it into contact with a substance, which contact may result in binding between the polypeptide or peptide and the substance. Binding may be determined by any of a number of techniques available in the art, both qualitative and quantitative.

In various aspects the present invention is concerned with provision of assays for substances which inhibit interaction between a polypeptide of the invention and another polypeptide or molecule able to interact with it.

Further assays are for substances which interact with or bind a polypeptide of the invention and/or modulate one or more of its activities.

One aspect of the present invention provides an assay which includes:

(a) bringing into contact a polypeptide or peptide according to the invention and a putative binding molecule or other test substance; and

(b) determining interaction or binding between the polypeptide or peptide and the test substance.

A substance which interacts with the polypeptide or peptide of the invention may be isolated and/or purified, manufactured and/or used to modulate its activity as discussed. A test substance found to be able to bind is a candidate modulator of activity, either inhibitor or promoter of activity, and can be tested for ability to modulate the activity.

A further aspect of the present invention provides an assay method which includes:

(a) bringing into contact a substance including a polypeptide or fragment, mutant, variant or derivative thereof, in accordance with the present invention, a substance including a fragment of a second polypeptide or a fragment, mutant, variant or derivative of a second molecule, which is able to bind the relevant polypeptide of the invention; and a test compound, under conditions in which in the absence of the test compound being an inhibitor, the two said substances interact;

(b) determining interaction between said substance.

It is not necessary to use the entire proteins for assays of the invention which test for binding between two molecules. Fragments may be generated and used in any suitable way known to those of skill in the art. Suitable ways of generating fragments include, but are not limited to, recombinant expression of a fragment from encoding DNA. Such fragments may be generated by taking encoding DNA, identifying suitable restriction enzyme recognition sites either side of the portion to be expressed, and cutting out said portion from the DNA. The portion may then be operably linked to a suitable promoter in a standard commercially available expression system. Another recombinant approach is to amplify the

relevant portion of the DNA with suitable PCR primers. Small fragments (e.g. up to about 20 or 30 amino acids) may also be generated using peptide synthesis methods which are well known in the art.

The precise format of the assay of the invention may be varied by those of skill in the art using routine skill and knowledge. For example, the interaction between the polypeptides may be studied *in vitro* by labelling one with a detectable label and bringing it into contact with the other which has been immobilised on a solid support. Suitable detectable labels include ^{35}S-methionine which may be incorporated into recombinantly produced peptides and polypeptides. Recombinantly produced peptides and polypeptides may also be expressed as a fusion protein containing an epitope which can be labelled with an antibody.

Fusion proteins may be generated that incorporate six histidine residues at either the N-terminus or C-terminus of the recombinant protein. Such a histidine tag may be used for purification of the protein by using commercially available columns which contain a metal ion, either nickel or cobalt (Clontech, Palo Alto, CA, USA). These tags also serve for detecting the protein using commercially available monoclonal

antibodies directed against the six histidine residues (Clontech, Palo Alto, CA, USA).

The protein which is immobilized on a solid support may be immobilized using an antibody against that protein bound to a solid support or via other technologies which are known *per se*. A preferred *in vitro* interaction may utilise a fusion protein including glutathione-S-transferase (GST). This may be immobilized on glutathione agarose beads. In an *in vitro* assay format of the type described above a test compound can be assayed by determining its ability to diminish the amount of labelled peptide or polypeptide which binds to the immobilized GST-fusion polypeptide. This may be determined by fractionating the glutathione-agarose beads by SDS-polyacrylamide gel electrophoresis. Alternatively, the beads may be rinsed to remove unbound protein and the amount of protein which has bound can be determined by counting the amount of label present in, for example, a suitable scintillation counter.

An assay according to the present invention may also take the form of an *in vivo* assay. The *in vivo* assay may be performed in a cell line such as a yeast strain in which the relevant polypeptides or peptides are expressed from one or more

vectors introduced into the cell.

A method of screening for a substance which modulates activity of a polypeptide may include contacting one or more test substances with the polypeptide in a suitable reaction medium, testing the activity of the treated polypeptide and comparing that activity with the activity of the polypeptide in comparable reaction medium untreated with the test substance or substances. A difference in activity between the treated and untreated polypeptides is indicative of a modulating effect of the relevant test substance or substances.

Combinatorial library technology (Schultz, JS (1996) Biotechnol. Prog. **12**:729-743) provides an efficient way of testing a potentially vast number of different substances for ability to modulate activity of a polypeptide. Prior to or as well as being screened for modulation of activity, test substances may be screened for ability to interact with the polypeptide, e.g. in a yeast two-hybrid system (which requires that both the polypeptide and the test substance can be expressed in yeast from encoding nucleic acid). This may be used as a coarse screen prior to testing a substance for actual ability to modulate activity of the polypeptide.

The amount of test substance or compound which may be added to an assay of the invention will normally be determined by trial and error depending upon the type of compound used. Typically, from about 0.01 to 100 nM concentrations of putative inhibitor compound may be used, for example from 0.1 to 10 nM. Greater concentrations may be used when a peptide is the test substance.

Compounds which may be used may be natural or synthetic chemical compounds used in drug screening programmes. Extracts of plants which contain several characterised or uncharacterised components may also be used. A further class of putative inhibitor compounds can be derived from the relevant polypeptide of the invention. Peptide fragments of from 5 to 40 amino acids, for example from 6 to 10 amino acids from the region of the relevant polypeptide responsible for interaction with another molecule, may be tested for their ability to disrupt such interaction.

Other candidate inhibitor compounds may be based on modelling the 3-dimensional structure of a polypeptide or peptide fragment and using rational drug design to provide potential inhibitor compounds with particular molecular shape, size and charge characteristics.

Important assay methods of the invention employ an animal model, such as a *Cig30*, *Ssc1* or *Ssc2* transgenic or knockout mouse or other rodent. Such animal models and their generation are discussed in detail below.

A further aspect of the present invention therefore provides an assay method which comprises:

(a) treating an animal model as disclosed herein with a test substance;

(b) determining the presence or absence of an effect on the animal as a result of the treatment with the test substance.

The nature of an effect when detected may be investigated. Potential end-points for detection include visual effects, effects determined immunologically or biochemically, and effects determined by means of determination of gene expression, for instance by means of Southern or Northern blotting of nucleic acid extracts or derivatives from appropriate cells. The skilled person is well aware of the need for control experiments and well able to design appropriate controls, both positive and negative. An end-point indicative of a positive result may be chosen in view of the therapeutic application in mind.

In certain embodiments of this aspect of the invention, the animal model is a knockout for one or more of *Cig30*, *Ssc1* and *Ssc2*, the animal generally being a rodent, preferably mouse.

The purpose of an assay employing an animal model in accordance with the invention may be for identifying or obtaining an agent with therapeutic potential in treatment of a disorder resulting from a defect in Cig30, *Ssc1* and/or *Ssc2* function. Such disorders may include any mentioned herein. Of particular interest are agents useful in treatment of a skin disorder (such as liquid pharmaceuticals or others suitable for topical application), or eye problems, for which a *Cig30* knockout mouse is well suited.

Further embodiments employ animal models in which heterologous *Cig30*, *Ssc1* and/or *Ssc2* sequences are expressed in the animal, for instance particular mutant sequences or human sequences (e.g. in a mouse in which endogenous *Cig30*, *Ssc1* and/or *Ssc2* has been knocked out). This allows for screening for inhibitors and other modulators of the relevant function. These may be useful in treatment of e.g. a disease or disorder mentioned herein.

An animal may be treated with a test substance at an

appropriate dosage, depending on the site of administration (e.g. topically or to the eye), any known potency of the substance, solubility and other factors routinely taken into account by those skilled in the art.

Similar assay methods may employ host cells transformed with nucleic acid of the invention and expressing a polypeptide of the invention, or may employ cells or cell lines derived from transgenic animals (including knock-outs) generated as described further elsewhere herein.

Following identification of a substance which modulates or affects polypeptide activity, the substance may be investigated further. Furthermore, it may be manufactured and/or used in preparation, i.e. manufacture or formulation, of a composition such as a medicament, pharmaceutical composition or drug. These may be administered to individuals.

Thus, the present invention extends in various aspects not only to a substance identified as a modulator of polypeptide activity, in accordance with what is disclosed herein, but also a pharmaceutical composition, medicament, drug or other composition comprising such a substance, a method comprising

administration of such a composition to a patient, e.g. for treatment (which may include preventative treatment) of a disorder disease, use of such a substance in manufacture of a composition for administration, e.g. for treatment of a disorder or disease, and a method of making a pharmaceutical composition comprising admixing such a substance with a pharmaceutically acceptable excipient, vehicle or carrier, and optionally other ingredients.

Disorders and diseases which may be treated in accordance with aspects of the present invention have been discussed and mentioned already herein.

A substance identified using as a modulator of polypeptide or promoter function may be peptide or non-peptide in nature. Non-peptide "small molecules" are often preferred for many in vivo pharmaceutical uses. Accordingly, a mimetic or mimick of the substance (particularly if a peptide) may be designed for pharmaceutical use. The designing of mimetics to a known pharmaceutically active compound is a known approach to the development of pharmaceuticals based on a "lead" compound. This might be desirable where the active compound is difficult or expensive to synthesise or where it is unsuitable for a particular method of administration, e.g. peptides are not

well suited as active agents for oral compositions as they tend to be quickly degraded by proteases in the alimentary canal. Mimetic design, synthesis and testing may be used to avoid randomly screening large number of molecules for a target property.

There are several steps commonly taken in the design of a mimetic from a compound having a given target property. Firstly, the particular parts of the compound that are critical and/or important in determining the target property are determined. In the case of a peptide, this can be done by systematically varying the amino acid residues in the peptide, e.g. by substituting each residue in turn. These parts or residues constituting the active region of the compound are known as its "pharmacophore".

Once the pharmacophore has been found, its structure is modelled to according its physical properties, e.g. stereochemistry, bonding, size and/or charge, using data from a range of sources, e.g. spectroscopic techniques, X-ray diffraction data and NMR. Computational analysis, similarity mapping (which models the charge and/or volume of a pharmacophore, rather than the bonding between atoms) and other techniques can be used in this modelling process.

In a variant of this approach, the three-dimensional structure of the ligand and its binding partner are modelled. This can be especially useful where the ligand and/or binding partner change conformation on binding, allowing the model to take account of this the design of the mimetic.

A template molecule is then selected onto which chemical groups which mimic the pharmacophore can be grafted. The template molecule and the chemical groups grafted on to it can conveniently be selected so that the mimetic is easy to synthesise, is likely to be pharmacologically acceptable, and does not degrade *in vivo*, while retaining the biological activity of the lead compound. The mimetic or mimetics found by this approach can then be screened to see whether they have the target property, or to what extent they exhibit it. Further optimisation or modification can then be carried out to arrive at one or more final mimetics for *in vivo* or clinical testing.

Mimetics of substances identified as having ability to modulate polypeptide or activity using a screening method as disclosed herein are included within the scope of the present invention. A polypeptide, peptide or substance able to modulate activity of a polypeptide according to the present

invention may be provided in a kit, e.g. sealed in a suitable container which protects its contents from the external environment. Such a kit may include instructions for use.

A convenient way of producing a polypeptide according to the present invention is to express nucleic acid encoding it, by use of the nucleic acid in an expression system. Accordingly, the present invention also encompasses a method of making a polypeptide (as disclosed), the method including expression from nucleic acid encoding the polypeptide (generally nucleic acid according to the invention). This may conveniently be achieved by growing a host cell in culture, containing such a vector, under appropriate conditions which cause or allow expression of the polypeptide. Polypeptides may also be expressed in *in vitro* systems, such as reticulocyte lysate.

Systems for cloning and expression of a polypeptide in a variety of different host cells are well known. Suitable host cells include bacteria, eukaryotic cells such as mammalian and yeast, and baculovirus systems. Mammalian cell lines available in the art for expression of a heterologous polypeptide include Chinese hamster ovary cells, HeLa cells, baby hamster kidney cells, COS cells and many others. A common, preferred bacterial host is *E. coli*. Suitable vectors

can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. Vectors may be plasmids, viral e.g. 'phage, or phagemid, as appropriate. For further details see, for example, Molecular Cloning: a Laboratory Manual: 2nd edition, Sambrook et al., 1989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in Current Protocols in Molecular Biology, Ausubel et al. eds., John Wiley & Sons, 1992.

Thus, a further aspect of the present invention provides a host cell containing nucleic acid as disclosed herein. The nucleic acid of the invention may be integrated into the genome (e.g. chromosome) of the host cell. Integration may be promoted by inclusion of sequences which promote recombination with the genome, in accordance with standard techniques. The nucleic acid may be on an extra-chromosomal vector within the cell.

A still further aspect provides a method which includes introducing the nucleic acid into a host cell. The introduction, which may (particularly for in vitro introduction) be generally referred to without limitation as "transformation", may employ any available technique. For eukaryotic cells, suitable techniques may include calcium phosphate transfection, DEAE-Dextran, electroporation, liposome-mediated transfection and transduction using retrovirus or other virus, e.g. vaccinia or, for insect cells, baculovirus. For bacterial cells, suitable techniques may include calcium chloride transformation, electroporation and transfection using bacteriophage.

Marker genes such as antibiotic resistance or sensitivity genes may be used in identifying clones containing nucleic acid of interest, as is well known in the art.

The introduction may be followed by causing or allowing expression from the nucleic acid, e.g. by culturing host cells (which may include cells actually transformed although more likely the cells will be descendants of the transformed cells) under conditions for expression of the gene, so that the encoded polypeptide is produced. If the polypeptide is expressed coupled to an appropriate signal leader peptide it

may be secreted from the cell into the culture medium. Following production by expression, a polypeptide may be isolated and/or purified from the host cell and/or culture medium, as the case may be, and subsequently used as desired, e.g. in the formulation of a composition which may include one or more additional components, such as a pharmaceutical composition which includes one or more pharmaceutically acceptable excipients, vehicles or carriers (e.g. see below).

Introduction of nucleic acid may take place *in vivo* by way of gene therapy, as discussed below. A host cell containing nucleic acid according to the present invention, e.g. as a result of introduction of the nucleic acid into the cell or into an ancestor of the cell and/or genetic alteration of the sequence endogenous to the cell or ancestor (which introduction or alteration may take place *in vivo* or *ex vivo*), may be comprised (e.g. in the soma) within an organism which is an animal, particularly a mammal, which may be human or non-human, such as rabbit, guinea pig, rat, mouse or other rodent, cat, dog, pig, sheep, goat, cattle or horse, or which is a bird, such as a chicken. Genetically modified or transgenic animals or birds comprising such a cell are also provided as further aspects of the present invention.

Thus, in various further aspects, the present invention provides a non-human animal with a *Cig30*, *Ssc1* or *Ssc2* transgene within its genome. The transgene may have the sequence of any of the isoforms identified herein or a mutant, derivative, allele or variant thereof as disclosed. In one preferred embodiment, a heterologous human sequence replaces the endogenous animal sequence. In other preferred embodiments, one or more copies of the human sequence are added to the animal genome.

Preferably the animal is a rodent, and most preferably mouse or rat.

This may have a therapeutic aim. (Gene therapy is discussed below.) The presence of a mutant, allele or variant sequence within cells of an organism, particularly when in place of a homologous endogenous sequence, may allow the organism to be used as a model in testing and/or studying the role of the gene or substances which modulate activity of the encoded polypeptide and/or promoter *in vitro* or are otherwise indicated to be of therapeutic potential.

Further important aspects of the present invention are based on the inventors' construction of animal models for disease.

Animal models for the relevant gene deficiency may be constructed using standard techniques for introducing mutations into an animal germ-line. In one example of this approach, using a mouse, a vector carrying an insertional mutation within the gene may be transfected into embryonic stem cells. A selectable marker, for example an antibiotic resistance gene such as neoR, may be included to facilitate selection of clones in which the mutant gene has replaced the endogenous wild type homologue. Such clones may be also be identified or further investigated by Southern blot hybridisation. The clones may then be expanded and cells injected into mouse blastocyst stage embryos. Mice in which the injected cells have contributed to the development of the mouse may be identified by Southern blotting. These chimeric mice may then be bred to produce mice which carry one copy of the mutation in the germ line. These heterozygous mutant animals may then be bred to produce mice carrying mutations in the gene homozygously. The mice having a heterozygous mutation in the gene may be a suitable model for human individuals having one copy of the gene mutated in the germ line who are at risk of developing a disorder or disease.

The invention therefore further provides a non-human transgenic animal which harbours at least one copy of a

transgene either homologously or nonhomologously integrated into a chromosomal location and encoding a heterologous polypeptide of the invention, e.g. human sequence, or a mutant, variant, derivative, or fragment, of a human or mouse sequence.

In a further aspect the invention provides a non-human transgenic animal which harbours one or more integrated constructs or targeted mutations that disrupt the function of endogenous *Cig30*, *Ssc1* and/or *Ssc2* genes. Such animals are referred to as "knock-outs", although it is not required by this aspect of the invention that function be totally ablated (this may be preferred). The invention provides a non-human animal with at least one inactivated endogenous *Cig30*, *Ssc1* or *Ssc2* allele, and which is preferably homozygous for inactivated *Cig30*, *Ssc1* and/or *Ssc2* alleles.

Various approaches for targeting constructs or mutations (generally deletions) to chromosomal locations are available in the art, and generally make use of homologous recombination between a target sequence in the chromosome and a region in a vector which is substantially complementary to the target sequence. Sequences flanking a target gene or a portion thereof may be employed, allowing for deletion of the target

gene or the relevant portion.

Transgenic mutations, including deletions, in a gene locus in accordance with the present invention may be detected using conventional techniques, such as fluorescent in situ hybridisation (FISH) with appropriate probes appropriately labelled, PCR analysis etc.

A transgenic animal according to the present invention is generally selected from mammals such as rabbit, guinea pig, rat, mouse or other rodent, cat, dog, pig, sheep, goat, cattle or horse, and preferably rodent, most preferably mouse.

Animal models may also be useful for any of the various diseases discussed elsewhere herein.

However, a non-obvious application based on the inventors' work is use in identifying and obtaining agents for treatment of eye problems, and skin disorders, which may be selected from disorders such as atopic dermatits, hair loss, psoriasis, cachexia and others disclosed herein and apparent to the skilled person based on the present disclosure. Assay methods employing animals in accordance with the present invention have been described already above.

Further aspects of the present invention provide cells of transgenic animals as disclosed, whether isolated cells or cell lines derived from the animals and optionally immortalised using standard techniques.

Host cells transformed with a polynucleotide of the invention provide further aspects of the invention, and are useful for example in production of an encoded polypeptide. Instead of or as well as being used for the production of a polypeptide encoded by a transgene, host cells may be used as a nucleic acid factory to replicate the nucleic acid of interest in order to generate large amounts of it. Multiple copies of nucleic acid of interest may be made within a cell when coupled to an amplifiable gene such as dihyrofolate reductase (DHFR), as is well known. Host cells transformed with nucleic acid of interest, or which are descended from host cells into which nucleic acid was introduced, may be cultured under suitable conditions, e.g. in a fermentor, taken from the culture and subjected to processing to purifiy the nucleic acid. Following purification, the nucleic acid or one or more fragments thereof may be used as desired, for instance in a diagnostic or prognostic assay as discussed elsewhere herein.

The provision of the novel polypeptides enables for the first

time the production of isolated antibodies able to bind these molecules specifically.

Accordingly, a further aspect of the present invention provides an antibody able to bind specifically to the polypeptide whose sequence is given in a figure herein. Such an antibody may be specific in the sense of being able to distinguish between the polypeptide it is able to bind and other human polypeptides for which it has no or substantially no binding affinity (e.g. a binding affinity of about 1000x less). Specific antibodies bind an epitope on the molecule which is either not present or is not accessible on other molecules. Antibodies according to the present invention may be specific for the wild-type polypeptide. Antibodies according to the invention may be specific for a particular mutant, variant, allele or derivative polypeptide as between that molecule and the wild-type polypeptide, so as to be useful in diagnostic and prognostic methods as discussed below. Antibodies are also useful in purifying the polypeptide or polypeptides to which they bind, e.g. following production by recombinant expression from encoding nucleic acid.

Preferred antibodies according to the invention are isolated,

in the sense of being free from contaminants such as antibodies able to bind other polypeptides and/or free of serum components. Monoclonal antibodies are preferred for some purposes, though polyclonal antibodies are within the scope of the present invention.

Antibodies may be obtained using techniques which are standard in the art. Methods of producing antibodies include immunising a mammal (e.g. mouse, rat, rabbit, horse, goat, sheep or monkey) with the protein or a fragment thereof. Antibodies may be obtained from immunised animals using any of a variety of techniques known in the art, and screened, preferably using binding of antibody to antigen of interest. For instance, Western blotting techniques or immunoprecipitation may be used (Armitage et al., 1992, Nature 357: 80-82). Isolation of antibodies and/or antibody-producing cells from an animal may be accompanied by a step of sacrificing the animal.

As an alternative or supplement to immunising a mammal with a peptide, an antibody specific for a protein may be obtained from a recombinantly produced library of expressed immunoglobulin variable domains, e.g. using lambda bacteriophage or filamentous bacteriophage which display

functional immunoglobulin binding domains on their surfaces; for instance see WO92/01047. The library may be naive, that is constructed from sequences obtained from an organism which has not been immunised with any of the proteins (or fragments), or may be one constructed using sequences obtained from an organism which has been exposed to the antigen of interest.

Suitable peptides for use in immunising an animal and/or isolating anti-Ssc1 or anti-Ssc2 antibody especially include C-terminal fragments.

Antibodies according to the present invention may be modified in a number of ways. Indeed the term "antibody" should be construed as covering any binding substance having a binding domain with the required specificity. Thus the invention covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, including synthetic molecules and molecules whose shape mimicks that of an antibody enabling it to bind an antigen or epitope.

Example antibody fragments, capable of binding an antigen or other binding partner are the Fab fragment consisting of the VL, VH, Cl and CH1 domains; the Fd fragment consisting of the

VH and CH1 domains; the Fv fragment consisting of the VL and VH domains of a single arm of an antibody; the dAb fragment which consists of a VH domain; isolated CDR regions and F(ab')2 fragments, a bivalent fragment including two Fab fragments linked by a disulphide bridge at the hinge region. Single chain Fv fragments are also included.

A hybridoma producing a monoclonal antibody according to the present invention may be subject to genetic mutation or other changes. It will further be understood by those skilled in the art that a monoclonal antibody can be subjected to the techniques of recombinant DNA technology to produce other antibodies or chimeric molecules which retain the specificity of the original antibody. Such techniques may involve introducing DNA encoding the immunoglobulin variable region, or the complementarity determining regions (CDRs), of an antibody to the constant regions, or constant regions plus framework regions, of a different immunoglobulin. See, for instance, EP184187A, GB 2188638A or EP-A-0239400. Cloning and expression of chimeric antibodies are described in EP-A-0120694 and EP-A-0125023.

Hybridomas capable of producing antibody with desired binding characteristics are within the scope of the present

invention, as are host cells, eukaryotic or prokaryotic, containing nucleic acid encoding antibodies (including antibody fragments) and capable of their expression. The invention also provides methods of production of the antibodies including growing a cell capable of producing the antibody under conditions in which the antibody is produced, and preferably secreted.

The reactivities of antibodies on a sample may be determined by any appropriate means. Tagging with individual reporter molecules is one possibility. The reporter molecules may directly or indirectly generate detectable, and preferably measurable, signals. The linkage of reporter molecules may be directly or indirectly, covalently, e.g. via a peptide bond or non-covalently. Linkage via a peptide bond may be as a result of recombinant expression of a gene fusion encoding antibody and reporter molecule.

One favoured mode is by covalent linkage of each antibody with an individual fluorochrome, phosphor or laser dye with spectrally isolated absorption or emission characteristics. Suitable fluorochromes include fluorescein, rhodamine, phycoerythrin and Texas Red. Suitable chromogenic dyes include diaminobenzidine.

Other reporters include macromolecular colloidal particles or particulate material such as latex beads that are coloured, magnetic or paramagnetic, and biologically or chemically active agents that can directly or indirectly cause detectable signals to be visually observed, electronically detected or otherwise recorded. These molecules may be enzymes which catalyse reactions that develop or change colours or cause changes in electrical properties, for example. They may be molecularly excitable, such that electronic transitions between energy states result in characteristic spectral absorptions or emissions. They may include chemical entities used in conjunction with biosensors. Biotin/avidin or biotin/streptavidin and alkaline phosphatase detection systems may be employed.

The mode of determining binding is not a feature of the present invention and those skilled in the art are able to choose a suitable mode according to their preference and general knowledge. Particular embodiments of antibodies according to the present invention include antibodies able to bind and/or which bind specifically, e.g. with an affinity of at least 10^{-7} M , to a peptides fragment of a polypeptide as disclosed herein, especially a C-terminal fragment.

Antibodies according to the present invention may be used in screening for the presence of a polypeptide, for example in a test sample containing cells or cell lysate as discussed, and may be used in purifying and/or isolating a polypeptide according to the present invention, for instance following production of the polypeptide by expression from encoding nucleic acid therefor. Antibodies may modulate the activity of the polypeptide to which they bind and so, if that polypeptide has a deleterious effect in an individual, may be useful in a therapeutic context (which may include prophylaxis).

An antibody may be provided in a kit, which may include instructions for use of the antibody, e.g. in determining the presence of a particular substance in a test sample. One or more other reagents may be included, such as labelling molecules, buffer solutions, elutants and so on. Reagents may be provided within containers which protect them from the external environment, such as a sealed vial.

Whether it is a polypeptide, antibody, peptide, nucleic acid molecule, small molecule or other pharmaceutically useful compound according to the present invention that is to be

given to an individual, administration is preferably in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors.

A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated.

Pharmaceutical compositions according to the present invention, and for use in accordance with the present invention, may include, in addition to active ingredient, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material will depend on the route of administration, which may be oral, or by

injection, e.g. cutaneous, subcutaneous or intravenous.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, or Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

Targeting therapies may be used to deliver the active agent

more specifically to certain types of cell, by the use of targeting systems such as antibody or cell specific ligands. Targeting may be desirable for a variety of reasons; for example if the agent is unacceptably toxic, or if it would otherwise require too high a dosage, or if it would not otherwise be able to enter the target cells.

Instead of administering an agent directly, it may be be produced in target cells by expression from an encoding gene introduced into the cells, e.g. in a viral vector (see below). The vector may be targeted to the specific cells to be treated, or it may contain regulatory elements which are switched on more or less selectively by the target cells. Viral vectors may be targeted using specific binding molecules, such as a sugar, glycolipid or protein such as an antibody or binding fragment thereof. Nucleic acid may be targeted by means of linkage to a protein ligand (such as an antibody or binding fragment thereof) via polylysine, with the ligand being specific for a receptor present on the surface of the target cells.

An agent may be administered in a precursor form, for conversion to an active form by an activating agent produced in, or targeted to, the cells to be treated. This type of

approach is sometimes known as ADEPT or VDEPT; the former involving targeting the activating agent to the cells by conjugation to a cell-specific antibody, while the latter involves producing the activating agent, e.g. an enzyme, in a vector by expression from encoding DNA in a viral vector (see for example, EP-A-415731 and WO 90/07936).

Nucleic acid according to the present invention, e.g. encoding the authentic biologically active Ssc1 or Ssc2 polypeptide or a functional fragment thereof, may be used in a method of gene therapy, to treat a patient who is unable to synthesize the active polypeptide or unable to synthesize it at the normal level, thereby providing the effect provided by the wild-type with the aim of treating and/or preventing one or more symptoms of a disorder or disease.

Vectors such as viral vectors have been used to introduce genes into a wide variety of different target cells. Typically the vectors are exposed to the target cells so that transfection can take place in a sufficient proportion of the cells to provide a useful therapeutic or prophylactic effect from the expression of the desired polypeptide. The transfected nucleic acid may be permanently incorporated into the genome of each of the targeted cells, providing long

lasting effect, or alternatively the treatment may have to be repeated periodically.

A variety of vectors, both viral vectors and plasmid vectors, are known in the art, see e.g. US Patent No. 5,252,479 and WO 93/07282. In particular, a number of viruses have been used as gene transfer vectors, including adenovirus, papovaviruses, such as SV40, vaccinia virus, herpesviruses, including HSV and EBV, and retroviruses, including gibbon ape leukaemia virus, Rous Sarcoma Virus, Venezualian equine enchephalitis virus, Moloney murine leukaemia virus and murine mammary tumourvirus. Many gene therapy protocols in the prior art have used disabled murine retroviruses.

Disabled virus vectors are produced in helper cell lines in which genes required for production of infectious viral particles are expressed. Helper cell lines are generally missing a sequence which is recognised by the mechanism which packages the viral genome and produce virions which contain no nucleic acid. A viral vector which contains an intact packaging signal along with the gene or other sequence to be delivered (e.g. encoding the polypeptide or a fragment thereof) is packaged in the helper cells into infectious virion particles, which may then be used for the gene

delivery.

Other known methods of introducing nucleic acid into cells include electroporation, calcium phosphate co-precipitation, mechanical techniques such as microinjection, transfer mediated by liposomes and direct DNA uptake and receptor-mediated DNA transfer. Liposomes can encapsulate RNA, DNA and virions for delivery to cells. Depending on factors such as pH, ionic strength and divalent cations being present, the composition of liposomes may be tailored for targeting of particular cells or tissues. Liposomes include phospholipids and may include lipids and steroids and the composition of each such component may be altered. Targeting of liposomes may also be achieved using a specific binding pair member such as an antibody or binding fragment thereof, a sugar or a glycolipid.

The aim of gene therapy using nucleic acid encoding the polypeptide, or an active portion thereof, is to increase the amount of the expression product of the nucleic acid in cells in which the level of the wild-type polypeptide is absent or present only at reduced levels. Such treatment may be therapeutic or prophylactic, particularly in the treatment of individuals known through screening or testing to have a

disease susceptibility allele and hence a predisposition to the disease.

Similar techiques may be used for anti-sense regulation of gene expression, e.g. targeting an antisense nucleic acid molecule to cells in which a mutant form of the gene is expressed, the aim being to reduce production of the mutant gene product. Other approaches to specific down-regulation of genes are well known, including the use of ribozymes designed to cleave specific nucleic acid sequences. Ribozymes are nuceic acid molecules, actually RNA, which specifically cleave single-stranded RNA, such as mRNA, at defined sequences, and their specificity can be engineered. Hammerhead ribozymes may be preferred because they recognise base sequences of about 11-18 bases in length, and so have greater specificity than ribozymes of the Tetrahymena type which recognise sequences of about 4 bases in length, though the latter type of ribozymes are useful in certain circumstances. References on the use of ribozymes include Marschall, et al. Cellular and Molecular Neurobiology, 1994. 14(5): 523; Hasselhoff, Nature 334: 585 (1988) and Cech, J. Amer. Med. Assn., 260: 3030 (1988).

Aspects of the present invention will now be illustrated with

reference to the accompanying figures described already above and experimental exemplification, by way of example and not limitation. Further aspects and embodiments will be apparent to those of ordinary skill in the art. All documents mentioned in this specification are hereby incorporated herein by reference.

EXAMPLE 1 - Cloning and characterisation of Ssc1 and Ssc2

Mouse and human cDNA sequences were cloned as described below in Materials and Methods.

The deduced SSC1 and SSC2 polypeptides consist of 279 and 292 amino acids respectively. Relevant similarities and identities are as follows, calculated using BLAST with standard algorithm parameters W, T and X and the BLOSUM62 matrix (Altschul, et al., *supra*): CIG30-SSC1 44% similarity, 34% identity; CIG30-SSC2 43% similarity and 31% identity; SSC1-SSC2 50% similarity and 37% identity. Human SSC1 is 95% similar to and 92% identical with mouse SSC1.

At the nucleic acid level, using the program BESTFIT with gap creation penalty of 50, gap extension penalty of 3 the following results were obtained:

mouse *Ssc1* and human *Ssc1* - 89% identity with quality 6397, quality ratio 7.947, length 805, no gaps;

mouse *Ssc1* and *Ssc2* - 59% identity with quality 586, ratio 2.071, length 286, one gap;

mouse *Ssc2* and human *Ssc1* - 61% identity with quality 633, quality ratio 2.035, length 320, 3 gaps;

mouse *Ssc1* and *Cig30* - 63% identity with quality 229, quality ratio 2.290, length 106, one gap;

mouse *Ssc2* and *Cig30* - 67% identity with quality 154, quality ratio 3.667, length 42, no gaps;

human *Ssc1* and mouse *Cig30* - 64% identity with quality 214, quality ratio 3.194, length 67, no gaps.

BESTFIT uses the local homology algorithm of Smith and Waterman (Advances in Applied Mathematics (1981) 2: 482-489) to find the best segment of similarity between two sequences. The quality score for the best alignment to any point is equal to the sum of the scoring matrix values of the matches in that alignment, less the gap creation penalty times the number of

gaps in that alignment, less the gap extension penalty times the total length of all gaps in that alignment.

Northern blotting was used to show the presence of *Ssc 1* in liver, brown adipose tissue (BAT), heart, kidney, skin, spleen testis, brain, stomach, lung, and skeletal muscle, with highest levels in stomach, lung, kidney and skin, and *Ssc2* mRNA in only testis and liver.

Computer analysis of the *Cig30* cDNA sequence suggests it to be a transmembrane glycoprotein with a size of about 30 kD and with five putative transmembrane domains.

Twelve tissues were analysed for expression of *Cig30* mRNA, but detectable levels were only found in liver, brown adipose tissue and skin, where the expression is affected during tissue development. In hepatectomized mice suppression in *Cig30* expression was found in the liver whereas in skin an increased expression during fur development was detected. In brown adipose tissue the *Cig30* expression was selectively elevated about 200 times when mice were exposed to a three day cold stress. A similar increase, although to a lesser extent, was brought about in two other conditions of brown fat activation, namely during perinatal development and after

cafeteria diet. The magnitude of *Cig30* mRNA induction in the cold could be mimicked almost fully by chronic norepinephrine treatment indicating that the gene expression is mainly modulated by catecholamines *in vivo*. However, in primary cultures of brown adipocytes, a synergistic action of both norepinephrine and glucocorticoids was required for full expression of the gene. See Tvrdik et al., J. Biol. Chem. (1997) 272: 31738-31746.

EXAMPLE 2 - Cig30, Ssc1 and Ssc2 function

Three yeast proteins (JO343, FEN1 and SUR4) are significantly homologous to each other (Revardel et al, Biochimica et Biophysica Acta (1995) 1263: 261-265) and Cig30: SUR4-FEN1 50.3%, SUR4-JO343 47.2%, FEN1-JO343 66.8%. The yeast genes are indicated to function as membrane-bound fatty acid elongases and designated ELO1 (JO343), ELO2 (FEN1) and ELO3 (SUR4), where ELO1 elongates fatty acids specifically up to 16 carbons, and ELO2 and ELO3 specifically elongate up to very-long-chain fatty acids, 24-carbons and 26-carbons respectively. Mutations in the corresponding genes give rise to disorders in several metabolic pathways, as well as disorganised cytoskeleton, impaired regulation of cell growth and budding deficiency.

Cig30, Ssc1 and Ssc2 were individually expressed in ELO1, ELO2 and ELO3 yeast cells. Complementation analysis revealed that Cig30 and Ssc2 are functional equivalents to ELO2, while Ssc1 is equivalent to ELO3: the corresponding genes were able to rescue phenotypic disorders in the respective mutants, namely their incapacity to grow on lactate and insensitivity to inhibitors of sterol synthesis.

The inventors investigated whether the mouse genes would revert SR31747 resistance of the Δ*sur4* and Δ*fen1* yeast mutants. As demonstrated earlier, yeast vegetative growth was inhibited by the sterol synthesis inhibitor SR31747 in a dose-dependent manner (Silve et al., 1996, *Mol. Cell Biol.* 16, 2719-2727). Growth of the parental yeast strain EMA6 was severely inhibited by 2 μM SR31747. Following disruption of either *SUR4* or *FEN1*, however, SR31747 tolerance was greatly enhanced and the resulting mutants were able to grow in the presence of >35 μM SR31747.

In a first series of experiments, the Δ*sur4* mutant was transformed with yeast expression plasmids containing the genes of interest, and selected colonies were scored for their ability to grow in increasing concentrations of SR31747. Control transformation *SUR4* restored SR31747 sensitivity to

the wild type levels, while *FEN1* overexpression had no effect. In this expression system, *Ssc1* clearly conferred SR31747 sensitivity, even though less well than *SUR4*, and inhibited growth of the Δ*sur4* mutant at SR31747 concentrations of 20-25 μM. Transformation with *Cig30* did not affect SR31747 tolerance in the mutant.

A second series of experiments performed in the Δ*fen1* mutant revealed that *FEN1* restored wild type SR31747 sensitivity, but also that *SUR4* overexpression conferred nearly as low level of SR31747 sensitivity as *FEN1*. Of the homologous mouse genes, *Cig30* consistently complemented best the Δ*fen1* mutant, conferring growth arrest at 15-20 μM SR31747, but *Ssc1* could also mediate SR31747 sensitivity at higher concentrations (25-30 μM). In addition to being resistant to SR31747, the Δ*sur4* mutant was also found to be unable to grow on glycerol/ethanol (Silve et al., 1996, *Mol. Cell Biol.* 16, 2719-2727). In good agreement with the above results, *SUR4* or *Ssc1* rescued the Δ*sur4* mutant's capacity to grow on glycerol/ethanol, while the other genes did not.

Ssc1 rescues lethality of the Δsur4Δfen1 double mutant

EMY22, a *sur4* mutant, was transformed to Ura+ with the plasmid

pSSC1. Transformant were crossed with EMA41, a *fen1* disruptant. Diploid was sporulated and spores analysed for uracile prototrophy, SR31747 resistance, *fen1* deletion - replacement with LEU2, and for *sur4* mutation by PCR followed by sequence analysis. Among 67 spores analysed, only 3 were disrupted for *FEN1*. Among the three, one was an aneuploid, and one contained a wild type allele of the *SUR4* gene. The last spore was resistant to 25 μM SR31747. PCR analysis confirmed that the *fen1* gene was disrupted, and sequence analysis revealed that *sur4* was mutated at the ATG starting codon, as for the original mutant.

To further test this double mutant strain, it was verified that the presence of the plasmid was required for growth. When the medium is supplemented with uracile, URA3 cells are sensitive to the analog 5 fluoroorotic acid (5FOA), whereas ura3 mutant are able to grow in the presence of this inhibitor. A Ura^{+} spore with a wild type allele of FEN1 and the double mutant sur4 fen1, Ura^{+} and therefore expressing SSC1, were incubated in the presence of 5FOA (1mg/ml) and uracil for 48 hours. Only the wild-type cells were able to proliferate in these conditions, whereas the double mutant neve grew. Therefore, SSC1 is required for the double mutant growth. Even in the presence of fatty acids C20, C22 and C24,

or ceramides, the double mutant was not able to grow in the presence of the analog.

Ssc1 is involved in biosynthesis of C26

Recently, strong evidence has been provided that *ELO1*, *FEN1/ELO2* and *SUR4/ELO3* are involved in fatty acid chain elongation. In particular, *FEN1/ELO2* and *SUR4/ELO3* were reported to be necessary for the synthesis of fatty acids of up to 24 and 26 carbons, respectively. As a result, Δ*sur4* and Δ*fen1* mutants had modified sphingolipid composition.

In view of this, the same range of yeast transformants used in the previous experiments were metabolically labelled with [^{3}H]serine, [^{3}H]sphinganine or [^{3}H]inositol, and sphingolipids were isolated and separated by thin-layer chromatography. No significant difference was seen in sphingolipid synthesis between wild-type and the Δ*fen1* strain. The Δ*sur4* mutant showed, however, a modified sphingolipid pattern as compared to the parental wild type strain. Most notably, the band corresponding to $M(IP)_2C$ was absent in the mutant. This band could not be restored by overexpression of the *FEN1* gene, but complementation with the wild type *SUR4* gene restored the normal sphingolipid composition. Transformation with *Ssc1*

also ameliorated sphingolipid synthesis in the *Δsur4Δfen1* double mutant. On the other hand, *Ssc2* or *Cig30* had no effect. As M(IP)$_2$C contains almost exclusively cerotoyl (26:0), this experiment shows that *Ssc1* is similar to *SUR4* in its capacity to catalyse the synthesis of cerotic acid.

EXAMPLE 3 - Ssc1 expression in myelin-deficient mutant mice

Fatty acid elongation activity in mammalian tissues has been mainly studied in brain and in liver. The microsomal elongation system, especially in the brain, has been extensively studied because the VLCFA are essential constituents of brain sphingolipids such as cerebrosides and sulfatides (J.M. Bourre et al., 1978, Adv. Exp. Med. Biol. 101, 17-26). However, the relative physiological significance of the elongation system is not fully elucidated.

In early studies, Sidman et al. (Sidman et al., 1964, Science 144, 309-311) described two separate mutant mice that were deficient in myelin throughout the CNS. One was described as an autosomal, recessive mutation and termed quaking (qk) whereas the other more severe myelin deficient mouse was an X-chromosome linked recessive mutation named jimpy (jp). In the jimpy mutation the primary genetic lesion resides in a defect

in the myelin proteolipid protein (PLP) gene. The primary molecular defect in quaking remains to be established. Analysis of the lipid composition of brain in the quaking mouse showed a marked decrease in the VLCFA (Baumann et al., 1968, Eur. J. Biochem. 4, 340-344) and it was later established that the brain microsomal fatty acid elongation system was deficient in providing the fatty acids necessary for myelin formation (Goldberg et al., 1973, Science 182, 497-499). A decrease in overall fatty acid chain elongation activity was observed in both the quaking and jimpy mouse brain microsomes relative to controls (Bourre et al., 1975, Biochem. Biophys. Res. Commun. 63, 1027-1034).

Ssc1 is marked downregulated in the brains of myelin-deficient mouse mutants Quaking and Jimpy

Ssc1 mRNA levels in the brains of 19-day-old *quaking* mutants were reduced by 40% as compared to their normal littermates, while the actin mRNA levels in the same mutants were only insignificantly reduced by 10%. In the *jimpy* mutant, cerebral *Ssc1* mRNA levels were downregulated even more dramatically, being reduced by 70%. Thus, the reduced fatty acid chain elongation activity in the brains of *quaking* and *jimpy* mutants appears to correlate with expression of the *Ssc1* gene.

EXAMPLE 4- Transgenic overexpression of Cig30

Cig30 overexpression in testis does not influence the mRNA level of Ssc2

The inventors generated a transgenic line of mice which expresses *Cig30* cDNA under CMV (Cytomegalovirus) promoter. Curiously, despite the broad specificity of CMV expression, the transgene was only expressed only in the testis and (more weakly) in ovaries. Mo morphological deviations were found on the electron microscopy level, and both the transgenic males and females were fertile. To address the possibility whether there was a feed-back inhibition of *Ssc2* transcription by ectopic expression of *Cig30*, Northern blot analysis of *Ssc2* mRNA levels was performed in the CMV-*Cig30* transgenic mice and in their non-transgenic littermates. Transgenic expression of *Cig30* did not exert any change in endogenous *Ssc2* mRNa levels, suggesting that there is no feed-back interaction between the two paralogous genes.

Cig30 mRNA induction coincides with enhanced fatty acid chain elongation activity during brown fat recruitment

Among the members of the novel mouse gene family described

here, the most dramatic regulatory change so far observed was that of *Cig30* gene expression during brown fat recruitment.

The inventors isolated microsomal membranes from brown fat of thermoneutral and cold stimulated mice, and determined fatty acid chain elongation capacity in these samples. The results show that elongation activity was indeed significantly increased in brown fat of mice stimulated by low ambient temperature (4°C) for 3 days, as compared to thermoneutral controls. The highest increase (4.7x) was observed when palmitoyl-CoA (16:0) was used as substrate. Lower values were obtained with arachidoyl-CoA (20:0) used as substrate (4.3x), whereas elongation activity on lignoceroyl-CoA (24:0) was not statistically different.

Hence, the data demonstrate that elongation of two acyl-CoA substrates is strongly enhanced in the recruiting brown fat, paralleling the induction of *Cig30* expression.

MATERIAL AND METHODS

Animals and treatments

For comparison of *Ssc1*, *Ssc2* and *Cig30* expression profiles and

for fatty acid chain elongation studies, NMRI male mice (6-8 weeks old) were obtained from the local supplier (Eklunds) and kept at thermoneutral temperature (28°C) for 1 week. After this period, some animals were exposed to 4°C when indicated. The mice were sacrificed by cervical dislocation and the tissues were dissected and directly subjected to RNA extraction or homogenisation and differential centrifugation.

To analyse cerebral *Ssc1* mRNA levels dysmyelinating mutants, heterozygous breeder pairs of Quaking (B6C3Fe-*a/a-qk*) and Jimpy (B6CBACa-A^{w-J}/A-*Ta jp)* were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and bred in an animal facility. The mutant pups and their healthy littermate controls were sacrificed at the age of 18 days and their brains were quickly dissected and frozen in liquid nitrogen before RNA extraction.

Transgenic mouse techniques

The CMV-*Cig30* transgenic line of mice was created by pronuclear microinjection according to the standard protocols (Hogan et al., (1986) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). The DNA construct was prepared by subcloning of a cDNA fragment corresponding to nt 162 to 1056

in the *Cig30* mRNA into *Xho*I and *Xba*I sites of the pCI-neo vector (Promega). The construct was digested with *Bgl*II and *Ngo*MI, and the 2.5-kb fragment containing the CMV promoter, chimeric intron, *Cig30* open reading frame and the synthetic polyadenylation signal was gel-purified and injected (0.2 ng/μl) into pronuclei of fertilised mouse eggs collected from 4-week-old C57BL/6J x CBA/J females (Bommice). Microinjected eggs were implanted at two-cell stage into oviducts of pseudopregnant foster mothers of the FVB/N strain. One founder was obtained, which was bred to establish a mouse line homozygous for the transgenic locus. The line expressed the transgene only in testis and, to a lesser degree, in ovaries.

Mouse cDNA cloning and sequencing

Primers were designed to PCR amplify the protein coding regions of *Ssc1*, *Ssc2* and *Cig30* from mouse liver Marathon-Ready cDNA library (Clontech). The primers used were:

Primer 1

5'-GGAC**GTCGAC**TGAGTCCTTAGCCAGGATGGAGGCTGTTGT-3', and

primer 2

5'-GAGC**AGATCT**GTCCTGAGGCACTTAGGTGGGCAATGTCTA-3',

for *Ssc1* ORF amplification;

primer 3

5'-GGAC**GTCGAC**CGCGGCCGCGCGGCCATGGAGCAGCTGAA-3', and

primer 4

5'-GAGC**AGATCT**CCACCTCAGTTTGTGTTCCCCGGCACTTCA-3',

for *Ssc2* ORF amplification;

primer 5

5'-GGAC**GTCGAC**CGTCTGCAAAATCGAAATGGACACATCCAT-3', and

primer 6

5'-GAGC**AGATCT**ACGGAGGAACGGCTGAGGCTCCATCTTTCT-3'.

for *Cig30* ORF amplification.

These may be useful for amplifying and cloning the human homologues.

Specific primers useful for cloning human *SSC1* are shown below.

To facilitate cloning, all forward primers contain exogenous *Sal*I sites and all reverse primers contain foreign *Bgl*II sites (both shown in bold face). The touch-down PCR reactions were performed with the *Pfu* polymerase (Stratagene) for 32 cycles totally (after denaturation at 94°C for 1 min, 5 cycles 94°C for 30 sec, 72°C for 3 min, 5 cycles 94°C for 30 sec, 70°C for

3 min, and 22 cycles 94°C for 30 sec, 68°C for 3 min). For each gene, at least two independent PCR products were sequenced to check that no amplification errors occurred.

In order to obtain the full-length mRNA sequences of *Ssc1* and *Ssc2*, RACE experiments were performed using:

primer 7

5'-GGCTATTGGAAAAGTCTATGGGGTCACA-3' for *Ssc1* 5' RACE,

primer 8

5'-GGCACCATCTTCTTCATACTGTTCTCCA-3' for *Ssc1* 3'RACE,

primer 9

5'-CCAGCATATACGCAGAAAGAAGTGTG-3' for *Ssc2* 5' RACE, and

primer 10

5'-GACATACCGGAAAAAGCCAGTGAAGAAA-3' for *Ssc2* 3' RACE.

The template and PCR conditions were same as above.

The PCR products were subcloned into pCR-XL-TOPO vector (Invitrogen) and sequenced using ABI Prisms Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer) with an ABI373A automatic DNA sequencer (Applied Biosystems).

Human *Ssc1* was cloned in an analogous fashion from a human liver Marthon-Ready cDNA library (Clontech). The following primers were employed:

Human-ssc1-90p

5'-GCCCCTACGGAGTCCTTAGCCA-3'

Human-ssc1-1007p

5'-GGTGCAGTCCTGAGGCACTTAGG-3'

RNA isolation and Northern blotting

Total RNA was isolated from fresh or frozen specimens of mouse tissues (ca 50mg) using the Ultraspec RNA isolation protocol (Biotech Laboratories). RNA electrophoresis, blotting and hybridisation was performed as previously described (Tvrdik et al., (1997) *J. Biol. Chem.* 272, 31738-31746). The cDNA probes for *Ssc1*, *Ssc2* and *Cig30* were *Sal*I-*Bgl*II fragments isolated from their corresponding pEMR yeast expression vectors. The actin probe was as in (Tvrdik et al., (1997) *J. Biol. Chem.* 272, 31738-31746). The probes were labelled using random-primed DNA labelling kit (Boehringer Mannheim) with [^{32}P]dCTP.

Yeast strains and culture conditions

Yeast strains used in this study and their genotypes are

presented in Table 1. They are all FL100 and FL200 derivatives. For metabolic labelling and growth studies, all strains were grown on YNBG medium (Difco), supplemented with tryptophan (20 mg/l) (for EMA41 transformants), tryptophane and leucine (for EMY58 transformants) and tryptophan and uracil (for EMY3).

Yeast plasmids and DNA manipulation

pEMR1023, a multicopy plasmid containing the selection marker URA3, was described previously (Silve et al., (1996) *Mol. Cell. Biol.* 16, 2719-2727). The coding sequence of *Ssc1, Ssc2* and *Cig30* were obtained by PCR amplification as described above, digested with *Sal*I and *Bgl*II, and ligated into *Sal*I and *Bgl*II sites of the pEMR1023 vector. Yeast transformation was carried out as described (Gietz et al., (1992) *Nucleic Acids Res* 20, 1425). The *SUR4* gene disruption was performed by replacing the 1.1kb fragment between the *Pvu*II and *Hpa*I sites of *SUR4* with a 1.4kb *Dra*I-*Mlu*I fragment encompassing the gene conferring geneticin resistance. The resulting 3-kb *Bam*HI cassette was used to replace the *SUR4* gene in EMY30 cells. Disruption was checked by PCR analysis. Disruptant cells were resistant to 200 μg/ml geneticin and 25 μM SR31747.

Sphingolipid analysis

Overnight cultures of yeast transformants were diluted to approx. 2 x 10^6 cells/ml (OD_{600}=0.066) and grown in 2 ml of appropriately supplemented YNBG at 30°C in the presence of the radioactive precursor. [^{3}H]serine (20 μCi/ml) was added immediately after dilution and the cells were labelled for 6 h. [^{3}H]sphinganine and [^{3}H]inositol (each at 1 μCi/ml in the total chemical concentration of 10 μM) were added 4.5 h after dilution and the cells were labelled for 1.5 h. Incubations were terminated by chilling on ice and 0.5 ml of unlabeled stationary phase cells was added. The cultures were sedimented at 2800 x *g* for 10 min at 4°C, treated with 5% TCA at 4°C for 20 min and washed once with 5 ml of ice-cold H_2O. Lipids were prepared as described (Hanson and Lester., (1980) *J Lipid Res* 21, 309-15). Briefly, each pellet was extracted twice with 1 ml of ethanol/water/diethyl ether/pyridine/4.2 N NH_4OH (15:15:5:1:0.018) at 60°C for 15 min. In order to destroy glycerophospholipids, the pooled extracts were optionally treated with 1 ml of monomethylamine reagent (33% monomethylamine in ethanol, diluted by 30%(v/v) with water) at 52°C for 30 min (Clarke and Dawson., (1981) *Biochem J* 195, 301-6). The extracts were then dried in SpeedVac and dissolved in 120 μl of chloroform/methanol/water (16:16:5)

Thirty μl of each sample were applied to Whatman LK5D silica gel TLC plates and resolved in chloroform/methanol/4.2NH_4OH(9:7:2). When the runs were completed, the plates were dried at 100°C for 5 min and sprayed several times with EN^3HANCE (NEN Life Science Products). The signal was visualised by exposing the TLC plates to DuPont Cronex X-ray films at -80°C for several weeks.

Preparation of microsomes and fatty acid elongation assay

Interscapular brown fat was dissected and homogenised in 4 ml of ice-cold 0.25 M sucrose. Following a 30-min stepwise centrifugation (10 min at each 700 *g*, 8000*g* and 17000 *g*) at 4 to 10°C, the supernatant was carefully transferred to fresh tubes and microsomes were sedimented at 105000 *g* for 45 min. The pellet was resuspended in 20 mM Tris-HCl, pH7.4, containing 0.4 M KCL, and centrifuged at 105000 *g* 45 min. The final microsomal pellet was resuspended in 200 μl of 0.1 M Tris-HCl, pH 7.4, and the protein was measured with the BCA protein assay (Pierce).

Total fatty acid elongation activity was measured essentially according to Suneja et al., (1991) *J. Neurochem.* 57, 140-146.

The assay mixtures (1 ml total, including protein addition) contained 0.1 M Tris-HCl, pH 7.4; either 50 μM palmitoyl-CoA, 15 μM arachidoyl-CoA or 15 μM lignoceroyl -CoA; substrate/BSA ratio 2:1; 1 mM NADPH; and 50 μM malonyl-CoA (containing 0.20 μCi of [2-^{14}C]malonyl-CoA). After 1 min of preincubation at 37°C, the reaction was initiated by the addition of 1 mg of microsomal protein and carried out for 20 min at 37°C. The incubation was terminated by addition of 1 ml of 15% KOH in methanol and saponified at 65°C for 45 min. Then the samples were cooled and acidified with 1 ml of cold 5 M HCl. Free fatty acid were extracted from the mixture three times with 3 ml of n-hexane and dried under vacuum. The extract was dissolved in 1 ml of chloroform and measured after addition of 10 ml of scintillation mixture in a Beckman liquid scintillation system 3801.

EXAMPLE 5 - Generation of Cig30 knockout mice

MATERIAL AND METHODS

Gene targeting vector

A genomic liver DNA library from mouse strain 129/Sv cloned into the Lambda FIXII vector was used to isolate a DNA

fragment containing the entire *Cig30* gene.

Mouse Cig30 genomic cloning

Genomic clones of *Cig30* were isolated by plaque hybridisation of a commercial mouse 129 strain liver genomic DNA library in the Lambda FIX II vector (Stratagene Catalogue # 946308) with a ^{32}P-labelled probe corresponding to 1.2 kb from the 5' end of the *Cig30* cDNA (GenBank U97107, Tvrdik et al. Figure 1a, *supra*). Hybridisation was carried out overnight at 45°C in 50% formamide, 5 x SSC, 5 x Denhardt's solution, 50 mM sodium phosphate, pH 6.5, 0.5% SDS and 100 μg/ml degraded herring sperm DNA. The membranes were first washed twice for 15 min at room temperature in 2 x SSC, 0.1% SDS, and a high stringency was then performed at 55°C in 0.1 x SSC, 0.1% SDS for 15-30 min. Phage DNA was prepared on a large scale by the PEG precipitation method. *Cig30* gene was checked by hybridisation with a 3' UTR probe (0.8 kb from the 3' end of the *Cig30* cDNA).

DNA sequencing and sequence analysis

Using recombinant phage DNA as a template, the genomic insert was sequenced by the primer walking strategy. Sequencing was

performed with an ABI373A automatic DNA sequencer (Applied Biosystems) on reactions prepared by the dye-termination method, using the ABI Prisms Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer). The sequence information was compiled and analysed with the use of the University of Wisconsin Genetics Computer Group software. The complete nucleotide sequence of the 13,869-bp genomic fragment has been deposited in GenBank (Accession No. AF054504).

The DNA probes used were as follows:

(i) the *Cig30* ORF probe - a 871-bp fragment corresponding to nt 162-1056 in the *Cig30* cDNA;

(ii) the *Cig30* 3' probe - a 294-bp *Pst*I-*Stu*I fragment corresponding to nt 10646-10939 in the genomic clone; and

(iii) the *Pitx*3 probe - a 897-bp *Pst*I-*Pst*I fragment corresponding to nt 11443-12340 in the genomic clone.

The probes were labelled with [α-^{32}P]dCTP using a random primed DNA labelling kit (Boehringer Mannheim).

A genomic liver DNA library from mouse strain 129/Sv cloned into the Lambda FIXII vector was used to isolate a DNA fragment containing the entire *Cig30* gene. A 2.75 kb fragment

between SacI and SalI upstream of the first exon was subcloned into the polylinker of the pBluescript SK(+/-) plasmid in order to become the left arm in the gene targeting vector. The SacI site was blunt-ended to T4 DNA polymerase (New England Biolab) and turned into a NotI site by ligation of NotI primers (Bio Source International). A 1.2 kb fragment containing a *neomycin^r (neo^r)* gene was cut out with XhoI from the plasmid KT1 LoxA (Thomas and Capecchi, (1987) *Cell* 51, 503-512) and inserted into the compatible SalI site of the plasmid containing the 2.75 kb left arm fragment. The ligation between SalI and XhoI ends consequently destroyed the recognition site for respective digestion enzyme. A 5.30 kb SalI/SalI fragment downstream of the second exon of *Cig30* was ligated into the pBluescript SK(+/-) plasmid and further digested with SalI and XhoI to receive a 2.77 kb fragment corresponding to the right arm in the gene targeting vector. This fragment was subcloned into the XhoI site of the vector containing the 2.75 kb left arm fragment and the *neo^r* gene. The NotI/XhoI fragment from the resulting plasmid was ligated into the corresponding sites in the TK1-TK2 vector (Barrow, J.R. and Capecchi, M.R. (1996) *Development* 122, 3817-3828).

Gene-targeting in ES cell cultures

The targeting vector was linearized with SalI and electroporated into the R1 embryonic stem (ES) cells (Nagy, A. *et al* (1993) *Proc. Natl. Acad, Sci. USA* 90, 8424-8428) derived from male 129/Sv agouti mice. ES cells were cultured as earlier described (Thomas, K.R. and Capecchi, M.R. (1987) *Cell* 51, 503-512) and exposed to 250 μg/ml G418 and 2 μM GANC (Gangcyclovir) as positive and negative selection respectively for three weeks (Mansour, S.L *et al* (1988) *Nature* 336, 348-352). G418/GANC resistant ES cells were screened for targeting events by Southern blot analysis (see below).

Generation and screening of Cig30 knock-out mice

Cig30 targeted ES cells were injected into C57BL/6J blastocysts and implanted into white foster mothers (F1, CBAxC57BL6) according to standard procedures (Gossler, A. *et al*, (1986) *Proc. Natl. Acad. Sci. USA* 83, 9065-9069). The male offspring being the most chimeric, approximately 80% agouti and 20% black in the coat colour, were bred with C57Bl/6J female in order to generate offspring heterozygous for the mutation. Genotypic analysis was performed by Southern blotting with ScaI and probing with a 897 bp PstI genomic right arm fragment (see under DNA analysis).

RNA analysis

Total RNA was isolated using Ultraspec (Biotech lab.) from 50-100 mg (w/w) of each tissue. For Northern blot analysis, 20 μg of total RNA was separated on a 1.2% (w/v) formaldehyde agarose gel and blotted onto Hybond-N membrane (Amersham) in 20 x SSC. The Hybond-N membrane was prehybridised with a solution containing 5 x SSC, 5 x Denhardt's, 0.5%, SDS, 50 mM sodium phosphate, 50% formamide and 100 μg/ml of degraded DNA from herring sperm (Sigma) at 45°C. After prehybridisation, the Hybond-N membrane was transferred to a similar solution containing the denatured probe. The hybridisation was carried out over night at 45°C. The Hybond-N membrane was then washed twice in 2 x SSC, 0.2% SDS at 30°C for 20-30 min each and then twice in 0.1 x SSC, 0.2% SDS at 50°C for 45 min. The filters were analysed on a Molecular Dynamics PhosphorImager with the ImageQuant program. When the same membrane was analysed for several mRNA species, the previous probe was removed by boiling the membrane twice for 20 min in 0.1% SDS solution.

DNA analysis

Genomic DNA was prepared from mouse tails by the simplified mammalian DNA isolation procedure published by Laird *et al.*

(Laird *et al.*, (1991) *Nucleic Acids Res.* 19, 4293-4293). Tail biopsies were collected from 3-week-old mice and used directly for DNA isolation. ES cell DNA was prepared according to standard procedures (Nagy, A. *et al* (1993) *Proc. Natl, Acad. Sci USA* 90, 8424-8428), DNA from ES cells were digested with XbaI and XbaI/SaII respectively and analysed with a 752 bp AhdI/SaII probe from the 2.75 kb left arm fragment. Two further probes, the 1.2 kb *neo*ʳ (XhoI/XhoI) isolated from KT1 LoxA plasmid and the *Cig30* probe which binds to *Cig30* downstream of exon 4, were used to confirm or exclude ES cell clones from the first screening. The DNA for the probes were purified according to the Jetsorb Gel extraction kit (Genomed Inc.) and were labelled with [α-^{32}P]dCTP using a random primed labelling kit (Boehringer Mannheim). Ten μg of digested DNA was separated on 0.8% agarose gel and transferred to Hybond-N membrane (Amersham) in 20 x SSC. The hybridisation procedure were identical to those for Northern blot analysis, except that hybridisation was carried out over night at 55°C. Hybond-N membrane was then washed twice in 2 x SSC, 0.2% SDS at 30°C for 20 min each and then once in 0.1 x SSC, 0.2% SDS at 55°C for 30 min. The filters were analysed on a Molecular Dynamics PhosphorImager with the ImageQuant program.

RESULTS

Targeting in ES cells

A *Cig30* genomic DNA fragment was initially isolated from a liver genomic lambda library (Figure 8). A 2.75 kb fragment downstream of the transcription start site was subcloned into a Bluescript vector as described in Materials and Methods. The following subcloning of the *neo*^{r} gene and a 2.77 kb fragment as the right arm fulfilled the criteria for a gene targeting vector against *Cig30*. By incorporating a negative selection marker, the *thymidine kinase* (*tk*) gene, it became possible to enrich for homologous recombination events (Figure 9). The gene targeting vector was linearized with SalI and electroporated into R1 ES cells (Nagy, A. *et al.* (1993), *Proc. Natl. Acad. Sci. USA* 90, 8424-8428). Upon selection on G418 and GANC resistance 144 ES cell clones were chosen for Southern blot analysis.

DNA from the ES cells were digested with XbaI and hybridised with a probe which binds upstream of the transcription start site but still resides in the left arm of the targeting vector. In case of a homologous recombination event, the probe should hybridise with two fragments, the wild-type fragment of 5.836 bp and the targeted fragment of 4.703 bp in

size which is due to the fact that the *neo*^{r} gene will specifically replace a 2.1 kb endogenous fragment including the transcription start site and the first two exons upon a correct targeting event (Figure 9B). Seventeen putative targeted clones were investigated further by extended Southern blot analysis.

This time, DNA from ES cell clones were digested with ScaI and ClaI respectively and hybridised with one probe specifically towards the *neo*^{r} gene and a 897bp genomic probe binding downstream of the right arm fragment (see Materials and Methods). Two of the seventeen ES clones were positive for the targeting event. Digestion with ScaI of one of these clones, 1h1, resulted in a distinct single fragment of 11.644 bp when hybridising with the *neo*^{r} probe. Fragments of other sizes were due to random integration of the gene targeting vector as in clone 2e5. Digestion with ClaI shows one fragment of 5.639 upon homologous recombination. The fragment size in the randomly integrated ES cells is unpredictable, since there is only one ClaI site within the *Cig30* gene.

When the same blot was probed with the *Cig30* genomic fragment downstream of the right arm it gave rise to two fragments of 11.644 bp (ScaI) and 5.639 bp (ClaI) respectively, i.e. the

same two fragments seen with the *neo*^{r} gene probe. The wild-type allele showed a distinct band of 5.603 bp which was not seen in the previous hybridisation with the *neo*^{r} probe. In summary, two targeted *Cig30* ES clones from the initial 144 clones were identified.

Cig30-deficient mice

A schematic picture of how the *Cig30*-deficient mice were obtained is illustrated in Figure 10. ES cell clones were injected into blastocysts from C57B1/6 and implanted into white foster mothers. The resulting male chimeras which were approximately 80% agouti and 20% black in the coat colour were bred with C57BL/6J females. The resulting heterozygote offspring were bred to homozygosity and DNA was isolated and genotypically analysed by Southern blot analysis. The homozygote disrupted *Cig30* mice were easily distinguished from wild-type and heterozygous mice by showing a single 11.644 bp fragment.

Phenotype of Cig30-deficient mice

In order to confirm the *Cig30* knock-out expression in mouse, Northern blot analysis were performed with total RNA isolated

from brown adipose tissue, skin and liver, i.e. organs which express *Cig30*, from both knock-out and wild-type mice kept at 30°C and 4°C for 6 days. Northern blot analysis showed there is no detectable *Cig30* mRNA in these organs in the *Cig30* knock-out mice at any temperature compared with wild-type mice where there is detectable amounts in all three tissues especially in cold induced brown adipose tissue. Mice heterozygous for *Cig30* show an expression pattern similar to wild-type litter mates indicating that the gene is recessive.

Cig30 knock-out mice show a retarded growth during the first four-five weeks of age which in dissection studies can be seen as less muscle mass and less subcutaneous white fat mass. After approximately two weeks of age there is a more obvious phenotypic distinction between knock-out and wild-type mice. The fur is tousled and there is a marked decrease in hair over the whole body. These observations are confirmed by histology studies of the skin. The hairs, hair follicles and associated sebaceous glands are less numerous and show a rudimentary and inactive appearance in the knock-out mice compared to control mice. Further, the skin of *Cig30*-deficient mice was several times thinner than in the control mice which is due a underdevelopment of dermis and all dermal structures (adnexa).

Another distinct phenotype is the closed the eye lids, i.e. the eye lids open at a later stage during growth and can even in some mice be closed after weaning.

However, as indicated, *Cig30*-deficient mice tolerate cold exposure as well as the control mice which implies that the function of the brown fact in these animals is intact. The UCP1 mRNA levels were found not to change upon cold stimulation in *Cig30* knock-out mice compared with wild-type mice. In addition, histological studies revealed no difference between brown fat tissue from knock-out and control animals.

Since *Cig30* is expressed in liver as well, total liver from *Cig30*-deficient mice was analysed histologically with no indicatives of a different phenotype than from control mice.

DISCUSSION

Cig30, *Ssc1* and *Ssc2* belong to a novel mammalian gene family which has been shown to be implicated in the elongation of VLCFA as well as in the formation of specific sphingolipids. Since *Cig30* and the other mammalian family members shows a tissue specific expression it can be assumed that there is a

special need for VLCFA of a specific length in different cells at a specific time. This is verified by the experiments described above in which the inventors constructed a gene targeting vector for *Cig30* to disrupt the gene in mice.

The method employed for disrupting *Cig30* was based on the Positive-Negative-Selection (PNS) method originally described by Capecchi and co-workers (Mansour, S.L. *et al* (1988) *Nature* 336, 348-352). The method can target and select for homologous recombination of any endogenous gene.

The gene targeting vector which has two fragments of 2.75 kb and 2.77 kb, the left and right arm respectively, flanking the *neo*^{r} gene, facilitates replacement of exon 1 and 2 with the *neo*^{r} gene (Figure 9). Two out of 144 ES cell lines had undergone homologous recombination, which was confirmed by Southern blot analysis. The most important factor determining targeting frequency is the length of the target homology. In a study by Hasty et al. there was a 200-fold increase in gene-targeting efficiency associated with an increase of homology from 1.3 to 6.8 kb for replacement vectors (Hasty, P. *et al* (1991) *Mol. Cell. Biol.* 11, 5586-5591).

In the work described here, the inventors were unable to

ascribe the significance of the *tk* selection. However, the use of the *tk* selection as described by Mansour et al. showed a 200-fold enrichment of ES cells that contained homologous vs. non-homologous integration of the target vector (Mansour, S.L. *et al* (1988) *Nature* 336, 348-352). Most groups however, have observed only a 5-20 fold enrichment with this method (Koller, B.H. *et al* (1992) *Annu. Rev. Immunol.* 10, 705-730).

Analysis of the targeted clones was by Southern blot analysis since the inventors were unable to successfully amplify PCR fragments corresponding to homologous recombination of DNA from targeted ES cells. Initial difficulties were also encountered with probes binding unspecifically to the genome and restriction enzymes, i.e. SalI and ClaI, being unable to digest in an eukaryotic environment. The left arm-probe of 752 bp was chosen in order to perform the first screening of the 144 clones resulting in 17 putative correctly targeted ES cell clones. A final screening of these 17 clones was achieved by hybridising the same membranes with the *neo*^{r} probe and a probe that binds outside and upstream of exon 4 in the targeting vector (*Cig30* probe). DNA from the remaining 17 clones was digested with ScaI and ClaI. Although ClaI is methylated in an eucaryotic environment as in the ES cells (Nelson, M. (1991) *Nucleic Acids Res.* 19 Suppl., 2045-2071),

still 10% of the DNA as seen on Southern blots were digested. By these means two ES cell clones with a disrupted *Cig30* gene were finally detected. Both hybridisations gave rise to the expected fragments, as well as to the extra fragment of 5.603 bp with the latter probe.

When mice heterozygote for the targeting event were bred, a Mendelian 1:2:1 distribution was observed. This implies that *Cig30* expression is not essential for prenatal development. The relevance of VLCFA in general is indisputable. Depending on their chain length and degree of unsaturation, they regulate the fluidity and other physical and chemical properties of the membrane such as cell-cell contact, transport over the membrane, activities of membrane-associated enzymes and key regulators of the eukaryotic stress response (signal transduction molecules). The stress response is particular interesting in view of the strong and selective induction of *Cig30* in recruiting brown adipose tissue, suggesting that synthesis of a specific VLCFA is required for tissue recruitment and/or cell proliferation. Multiple lines of evidence are beginning to implicate sphingolipids, ceramides and sphingosines as key regulators of the eucaryotic stress response, e.g. ceramides formed after activation of sphingomyelinase (by TNF-α and IL-1) decrease cell division

and induce apoptosis (Kim, M.Y. *et al* (1991) *J. Biol. Chem.* 266, 484-489; Obeid, L.M. *et al* (1993) *Science* 259, 1769-1771) which on the other hand can be counteracted by sphingosine-phosphate which stimulate cell growth and inhibit apoptosis (Zhang, H. *et al* (1991) *J. Cell Biol.* 114, 155-167). A marked increase can be seen in the microsomal elongation activity of long chain and very long chain fatty acids from cold stimulated brown adipose tissue compared with warm controls.

The work clearly demonstrates involvement of VLCFA in skin development. Skin lipids are an important determinant for both water-retention function and permeability-barrier function. Sphingolipids (ceramides) have been reported to comprise up to 50% of the total amount lipids in epidermis which is the highest amount present in any mammalian tissue (Forslind, B. (1997) *J. Dermatol. Sci.* 14, 115-125). Another intriguing fact is that more than one-third of human stratum corneum lipids have chain lengths longer than 22-carbons, i.e. VLCFAs. Disorders such as atopic dermatitis correspond to impaired barrier function due to reduced ceramide content which leads to dry and irritated skin (Di Nardo, A. *et al* (1998) *Acta Derm. Venereol.* 78, 27-30).

The alterations seen in the *Cig30* knock-out mice may be regarded as a dysplastic skin decease, characterised by abnormal growth and development of the dermis and adnexa. Alternatively, the changes observed could be considered as an atrophic disease of the skin characterised by hair growth arrest. The skin changes observed in *Cig30*-deficient mice are most dramatic within the dermis layer, although it is not clear yet whether the skin barrier in itself is defect. Further analysis of the skin will reveal which cells are normally expressing *Cig30* and the spectrum of VLCFA and sphingolipids in these mice.

The mice show impaired hair growth, dry skin and much less subcutaneous fat than normal mice, and this appears to be due to a general developmental problem of the skin layers. However, despite the high *Cig30* expression in brown fat in wild-type mice during cold exposure, the *Cig30* knockout mice are fully capable of maintaining their thermoregulatory function including high UCP1 expression upon cold exposure.

The mice also show eye problems within the first 3-5 weeks after birth. This is thought to be due to undeveloped membonious glands in their eye lids which normally "oil" the eye so the lid is smoothly mobile. This provides for testing

of substances for treatment of eye disorders or other problems (e.g. when contact lenses are used), for instance ceramides with specific fatty acids.

CONCLUDING DISCUSSION

The inventors have characterized three paralogous genes in mice and homologues in humans, which genes are indicated to be the first mammalian genes directly involved in fatty acid elongation.

Cig30 was previously isolated in the laboratory as a gene specifically expressed during brown fat recruitment, and in liver and skin. The inventors have now identified and cloned two new full-length cDNA's, *Ssc1* and *Ssc2*. Analysis of expression in mice revealed that *Ssc1* mRNA is ubiquitously expressed in all tissues tested, with highest levels in stomach, lung, kidney, and skin. In contrast, *Ssc2* mRNA was found to be rather tissue specific, being predominantly expressed in testis and liver. The deduced SSC1 and SSC2 polypeptides are approximately 30% identical with CIG30. Rescue experiments of phenotypes of yeast null mutants demonstrate involvement in biosynthesis of very long chain fatty acids and consequently the formation of sphingolipids.

Cig30 deficient mice have been made and these have markedly diminished subcutaneous fat and abnormal hair growth. This is correlated with suppressed development of all epidermal and dermal structures.

The present invention allows for screening of agents for identification of candidate pharmaceuticals and other active agents, which may for instance be used in treatment of atopic dermatitis and other skin disorders, or in cosmetic applications, for instance in improving hair growth, or in treating eye problems.

Furthermore, *Ssc1* mRNA levels were found to be markedly reduced in brains of myelin-deficient mice which have decreased cerebral fatty acid chain elongation activity. This provides for screening for agents which may be used in treatment of MS.

Ssc2 was found to be highly expressed in testis, providing indication of a role in fertility given that ceramides (sphingolipids) are essential for function of sperm.

TABLE 1.

Yeast strains used in this study	
EMY58	Matα ura3 trp1 leu2 sur4::GenR (for SR31747 resistance)
EMA41	Mata ura3 trp1 leu2 fen1::LEU2 (for SR31747 resistance and crosses
EMA3	Mata ura3 trp1 (for SR31747 resistance)
EMY30	Matα ura3 trp1 leu2 (for SUR4 disruption)
EMY22	Matα ura3 sur4-232 (for crosses)

All these strains are FL100 or FL200 derivatives.

CLAIMS:

1. A non-human transgenic animal which is a knock-out for nucleic acid encoding a polypeptide which has at least 80% amino acid sequence identity with a polypeptide selected from

i CIG30, of which the amino acid sequence is shown in Tvrdik et al., J. Biol. Chem. (1997) 272: 31738-31746;

ii human SSC1, of which the amino acid sequence is shown in Figure 4;

iii mouse SSC1, of which the amino acid sequence is shown in Figure 2;

iv mouse SSC2, of which the amino acid sequence is shown Figure 6.

2. A non-human transgenic animal which has at least one copy of a polynucleotide according to any one of claims 28 to 30 below which is heterologous to said animal integrated into a chromosomal location.

3. An animal according to claim 1 or claim 2 that is a mouse or other rodent.

4. An assay method for obtaining an agent able to modulate fatty acid elongation, the method comprising contacting an

animal according to any one of claims 1 to 3 with a test compound, and determining ability of the test compound to affect a tissue of the animal.

5. An assay method for obtaining an agent that is a candidate pharmaceutical for use in therapeutic treatment of a skin or hair disorder or eye problem, the method comprising contacting an animal according to any one of claims 1 to 3 with a test compound and determining ability of the test compound to affect the skin, hair or eyes of the animal.

6. A method comprising:

(a) performing an assay method according to claim 4 or claim 5 to obtain a said agent;

(b) formulating said agent into a composition which comprises one or more additional components.

7. An agent obtained by a method according to claim 4 or claim 5.

8. Use of an animal according to any one of claims 1 to 3 for identifying a substance which modulates fatty acid elongation.

9. Use of an animal according to any one of claims 1 to 3 for identifying a substance which has a therapeutic effect on a disorder of the skin, hair or eyes.

10. An isolated polypeptide comprising an amino acid sequence selected from the group consisting of:

(1) the human Ssc1 amino acid sequence shown in Figure 4;

(2) the mouse Ssc1 amino acid sequence shown in Figure 2;

(3) the mouse Ssc2 amino acid sequence shown Figure 6.

11. An isolated polypeptide according to claim 10 consisting of the amino acid sequence shown in Figure 4, Figure 2 or Figure 6.

12. An isolated polypeptide which has an amino acid sequence which shares at least 80% identity with a polypeptide according to claim 11.

13. An isolated polypeptide which has an amino acid sequence which shares at least 90% identity with a polypeptide according to claim 11.

14. An isolated fragment, active portion, variant or derivative of a polypeptide according to claim 11, which has

one or more of the following properties:

immunological cross-reactivity with an antibody reactive with said polypeptide;

sharing an epitope with said polypeptide;

a biological activity which is inhibited by an antibody raised against said polypeptide;

ability to complement ELO1, ELO2 and/or ELO3 mutations in yeast;

enzymatic activity in common with said human Ssc1, mouse Ssc1 and/or mouse Ssc2.

15. An isolated fragment of a polypeptide according to claim 11, which fragment is at least 5 amino acids in length.

16. An isolated fragment according to claim 15 which is less than about 40 amino acids in length.

17. An assay method for obtaining an agent able to interact with a polypeptide, fragment, active portion, variant or derivative according to any one of claims 10 to 16, the method comprising:

(a) bringing into contact a substance which comprises a said polypeptide, fragment, active portion, variant or derivative , and a test compound; and

(b) determining interaction between said substance and the test compound.

18. An assay method for obtaining an agent with ability to modulate interaction between a polypeptide, fragment, active portion, variant or derivative according to any one of claims 10 to 16, and a second molecule, the method comprising:

(a) bringing into contact a substance which comprises said polypeptide, fragment, active portion, variant or derivative, a substance comprising a molecule which interacts with said polypeptide, fragment, active portion, variant or derivative, and a test compound; and

(b) determining interaction between said substances.

19. An assay method according to claim 17 or claim 18, the method further comprising determining ability of a test compound or an agent obtained in the assay to affect fatty acid elongation.

20. An assay method for obtaining an agent able to affect fatty acid elongation, the method comprising:

(a) bringing into contact a substance which comprises a polypeptide, fragment, active portion, variant or derivative according to any one of claims 10 to 16, and a test compound;

and

(b) determining fatty acid elongation activity.

21. An assay method according to any one of claims 17 to 20 further comprising determining ability of a test compound or agent obtained in the assay to affect mammalian skin, hair or eyes.

22. A method comprising:

(a) performing an assay method according to any one of claims 17 to 21 to obtain a said agent;

(b) formulating said agent into a composition which comprises one or more additional components.

23. An agent obtained by a method according to any one of claims 17 to 21.

24. Use of a polypeptide, fragment, active portion, variant or derivative according to any one of claims 10 to 16, for identifying a substance which interacts with said a polypeptide, fragment, active portion, variant or derivative.

25. Use of a polypeptide, fragment, active portion, variant or derivative according to any one of claims 10 to 16, for

identifying a substance which modulates fatty acid elongation.

26. Use of a polypeptide, fragment, active portion, variant or derivative according to any one of claims 10 to 16, for identifying a substance which has a therapeutic effect on a disorder of mammalian skin, hair or eyes.

27. An isolated specific binding member comprising an antigen-binding domain of an antibody specific for a polypeptide according to claim 10.

28. An isolated polynucleotide encoding a polypeptide according to any one of claims 10 to 13.

29. An isolated polynucleotide according to claim 28 of which the sequence encoding said polypeptide is shown in Figure 3, Figure 1 or Figure 5.

30. An isolated polynucleotide encoding a fragment, active portion, variant or derivative according to any one of claims 14 to 16.

31. An expression vector comprising a polynucleotide according to claim 29 or claim 30 operably linked to

regulatory sequences for expression of said polypeptide, fragment, active portion, variant or derivative.

32. A host cell transformed with an expression vector according to claim 31.

33. A method of making a product, the method comprising culturing a host cell according to claim 32 under conditions for expression of said polypeptide, fragment, active portion, variant or derivative to provide said product, and isolating or purifying said product.

34. A method according to claim 33, further comprising testing the product for ability to modulate fatty acid elongation.

35. A method according to claim 33 or claim 34, further comprising testing the product for therapeutic effect on a disorder of mammalian skin, hair or eyes.

36. A method according to any one of claims 33 to 35 wherein the isolated or purified product is formulated into a composition comprising one or more additional components.

37. A method which comprises determining in a sample the presence or absence of a polypeptide, fragment, active portion, variant or derivative according to any one of claims 10 to 16.

38. A method which comprises determining in a sample the presence or absence of nucleic acid encoding a polypeptide, fragment, active portion, variant or derivative according to any one of claims 10 to 16.

GATAGTTGTAAGTTTTTCTTTTTAAGAAAGCTCAACATTCTGCAGGCGGC
CCTGGGGACTTCTCTCCGGCCCTGATCCCTTTGAACCCTTCACTGCTGCC
CGCCTGGTTTCCACTGAGTCCTTAGCCAGGATGGAGGCTGTTGTGAACTT
GTACCACGAGCTGATGAAGCATGCGGATCCCCGGATCCAAAGCTACCCTC
TGATGGGGTCCCCCTTGCTAATAACATCCATCCTTCTGACCTATGTGTAC
TTCATCCTATCACTTGGGCCTCGAATCATGGCTAATCGGAAGCCCTTCCA
ACTTCGAGGCTTCATGATTGTCTACAATTTCTCACTGGTGATACTCTCCC
TCTACATTGTCTATGAGTTTCTGATGTCTGGTTGGCTGAGTACCTACACC
TGGCGCTGTGACCCCATAGACTTTTCCAATAGCCCTGAAGCACTTCGGAT
GGTTCGAGTGGCCTGGCTCTTCATGCTTTCCAAGGTCATTGAGCTGATGG
ACACAGTGATATTTATCCTCCGGAAGAAGGACGGGCAAGTGACCTTCCTC
CATGTCTTCCACCACTCGGTGCTTCCCTGGAGTTGGTGGTGGGGGATAAA
AATTGCTCCAGGAGGAATGGGCTCCTTCCATGCCATGATAAACTCCTCTG
TACATGTCGTCATGTACCTCTACTATGGATTGTCTGCCCTTGGCCCTGTG
GCCCAGCCCTACCTTTGGTGGAAGAAACATATGACTGCCATTCAGCTGAT
CCAGTTTGTCCTGGTCTCACTGCACATCAGCCAATACTACTTCATGCCCA
GCTGCAACTACCAGTACCCCATCATCATCCACCTCATCTGGATGTATGGC
ACCATCTTCTTCATACTGTTCTCCAATTTCTGGTATCACTCTTACACCAA
GGGGAAGCGGCTGCCCCGTGCAGTTCAGCAAAATGGAGCTCCAGCTACCA
CCAAGGTCAAGGCCAACTGAGAAGCATGGCCTAGACATTGCCCACCTAAG
TGCCTCAGGACTGCACCTTGGGCAGCATCCACAAGTGTCCTCTCTACCTA
CACCCGTGACCAGGGCTCTGTGGTCAGGACTGAGCAGGCGGACTGGCTCT
CCCCTCCCCACAGCTGGTCCACAGGGGCCACTGTTCCCATCCCTTCTCCC
AGCCAGCTCCAGGGATGTGGCCTCACTGCTGTCTGCCACCAGAACTGGAG
GCTGAAAGGGCTGGACACTTACTTCCCCCTCCCTGCCTTAAACTTGGGAG
AGGAACACTCAGGGCTGGCCGGCACGCACCCGGGTCTTGTGGCCTTTCTC
CTCACACTGAAGAGGTCAGCAATAATCTGTCACTGTGGACTCAGGCTCCC
TTCTCACTCCACATGGAAGCATGAGCTTCGGGCCAAAGGTCAGAATGGGC
AGGCAGCAGGCCTGGAAGTCAGGCTGTGCGCTCACTTGTGTCTTAATTAA
AGTGACGGGAAACCACTG

FIGURE 1

MEAVVNLYHELMKHADPRIQSYPLMGSPLLITSILLTYVVYFILSLGPRIM
ANRKPFQLRGFMIVYNFSLVILSLYIVYEFLMSGWLSTYTWRCDPIDFSN
SPEALRMVRVAWLFMLSKVIELMDTVIFILRKKDGQVTFLHVFHHSVLPW
SWWWGIKIAPGGMGSFHAMINSSVHVVMYLYYGLSALGPVAQPYLWWKKH
MTAIQLIQFVLVSLHISQYYFMPSCNYQYPIIIHLIWMYGTIFFILFSNF
WYHSYTKGKRLPRAVQQNGAPATTKVKAN*

FIGURE 2

TTGCCCCTACGGAGTCCTTAGCCAGGATGGAGGCTGTTGTGAACTTGTAC
CAAGAGGTGATGAAGCACGCAGATCCCCGGATCCAGGGCTACCCTCTGAT
GGGGTCCCCCTTGCTAATGACCTCCATTCTCCTGACCTACGTGTACTTCG
TTCTCTCACTTGGGCCTCGCATCATGGCTAATCGGAAGCCCTTCCAGCTC
CGTGGCTTCATGATTGTCTACAACTTCTCACTGGTGGCACTCTCCCTTCA
CATTGTCTATGAGTTCCTGATGTCGGGCTGGCTGAGCACCTATACCTGGC
GCTGTGACCCTGTGGACTATTCCAACAGCCCTGAGGCACTTAGGATGGtT
CGGGTGGCCTGGCTCTTCCTCTTCTCCAAGTTCATTGAGCTGATGGACAC
AGTGATCTTTATTCTCCGAAAGAAAGACGGGCAGGTGACCTTCCTACATG
TCTTCCATCACTCTGTGCTTCCCTGGAGCTGGTGGTGGGGGTAAAGATT
GCCCCGGGAGGAATGGGCTCTTTCCATGCCATGATAAACTCTTCCGTGCA
TGTCATAATGTACCTGTACTACGGATTATCTGCCTTTGGCCCTGTGGCAC
AACCCTACCTTTGGTGGAAAAAGCACATGACAGCCATTCAGCTGATCCAG
TTTGTCCTGGTCTCACTGCACATCTCCCAGTACTACTTTATGTCCAGCTG
TAACTACCAGTACCCAGTCATTATTCACCTCATCTGGATGTATGGCACCA
TCTTCTTCATGCTGTTCTCCAACTTCTGGTATCACTCTTATACCAAGGGC
AAGCGGCTGCCCCGTGCACTTCAGCAAAATGGAGCTCCAGGTATTGCCAA
GGTCAAGGCCAACTGAGAAGCATGGCCTAGATAGGCGCCCACCTAAGTGC
CTCAGGACTGCACCAAGGGCGAATTCAGGCCTGAATTCCAGCACACTGGC
GGCCGTTACTAGTGGATCC

FIGURE 3

MEAVVNLYQEVMKHADPRIQGYPLMGSPLLMTSILLTYVYFVLSLGPRIM
ANRKPFQLRGFMIVYNFSLVALSLYIVYEFLMSGWLSTYTWRCDPVDYSN
SPEALRMVRVAWLFLFSKFIELMDTVIFILRKKDGQVTFLHVFHHSVLPW
SWWWGVKIAPGGMGSFHAMINSSVHVIMYLYYGLSAFGPVAQPYLWWKKH
MTAIQLIQFVLVSLHISQYYFMSSCNYQYPVIIHLIWMYGTIFFMLFSNF
WYHSYTKGKRLPRALQQNGAPGIAKVKAN*

FIGURE 4

CGGCTACCCTGGACAGCGCATCGCGGCCGCGCGGCCATGGAGCAGCTGAA
GGCCTTTGATAATGAAGTCAATGCTTTCTTGGACAACATGTTTGGACCAC
GAGATTCTCGAGTTCGCGGGTGGTTCCTGCTGGACTCTTACCTTCCCACC
TTCATCCTCACCATCACGTACCTGCTCTCGATATGGCTGGGTAACAAGTA
CATGAAGAACAGGCCTGCTCTGTCTCTCAGGGGCATCCTCACCTTGTATA
ACCTCGCAATCACACTTCTTTCTGCGTATATGCTGGTGGAGCTCATCCTC
TCCAGCTGGGAAGGAGGTTACAACTTGCAGTGTCAGAATCTCGACAGTGC
AGGAGAAGGTGATGTCCGGGTAGCCAAGGTCTTGTGGTGGTACTACTTCT
CCAAACTAGTGGAGTTCCTGGACACGATTTTCTTTGTTCTACGAAAAAAG
ACCAATCAGATCACCTTCCTTCATGTCTATCACCACGCGTCCATGTTCAA
CATCTGGTGGTGTGTTTTGAACTGGATACCTTGTGGTCAAAGCTTCTTTG
GACCCACCCTGAACAGCTTTATCCACATTCTCATGTACTCCTACTACGGC
CTGTCTGTGTTCCCGTCCATGCACAAGTACCTTTGGTGGAAGAAGTACCT
CACACAGGCTCAGCTGGTGCAGTTCGTACTCACCATCACGCACACGCTGA
GTGCCGTGGTGAAGCCCTGTGGCTTCCCCTTTGGCTGTCTCATCTTCCAG
TCTTCCTATATGATGACGCTGGTCATCCTGTTCTTAAACTTCTATATTCA
GACATACCGGAAAAAGCCAGTGAAGAAAGAGCTGCAAGAGAAAGAAGTGA
AGAATGGTTTCCCCAAAGCCCACTTAATTGTGGCTAATGGCATGACGGAC
AAGAAGGCTCAATAAAATGAAGTGCCGGGGAACACAAACTGAGGTGGTGG
CGGCGGCGGCGGCAGCAAACAGACGAGCTTGTTTTAAAGCAGAGACT
GAATAGAAAGTTGTATGTTTTAGCATAAACTAATTCCTTTTGAGTTTGTA
AATCATTTGTACCCAGAATGTATTATAATATATTGCTATTAGGTTACTCT
ACTAACTGGAGCCATGCCGACCTCTACAAACCTTGAACAGGTGCACAGCC
CCCTCTCCGGAGCGCTTTGTAATGCCTTATTCACGTCGAAAACCAGGAGA
CTCGGGCTTCGTTTCTTCATGGCAAGTCTTCAGAGTTAATTTTCTTTAGA
TGTGTAAAACTGTTCATTGTAGTGTAAATGTCAGGGTCTGTTCTAGTCTA
TGTCAAGCAATAACTGTCAGTGCATAAATCGATTAGTCTGCTGTTGCTTA
AGGCAGCAGCCCCAGGCAGCATTTAAGCGGGATCCTTAGCAAGACAGAAT
CCAGCAAAACCCTTTCCCCTCTCAAGCCAGGAGTCTCATCTGCTACATTT
TTTTTAACCACCTCTGATTTCCAACTTAGTGATGTGGTAATGAAATTCGT
TTGTCTTCAATATAGTTTGAAGATTAGTCATTCCATGAAGGAACAGCCTC
TTGAGCTGGGTATGGTGGCACCCATCTGTGAGTCCAGCATTTGGGTATTG
AGACAGCCTAGGGTACACAGGCACAAGACCCTGTCTCAACCAGAAAAGAA
AAATGTGTTTGCCAAATCATAACTCAGGGGCTTTGAACAGAGCTGTCTCT
GTTTCAATTGGCATCCTTGCTCATCCATTCCTGTCTACCTCCCTTGTTAG
TCTAGATTTCTGGAGTTATGTTCCCCACATCCAAAGCCAGTGTCTACATA
CAGCTGCCATGCCCTTTCTGAACAAAGGCTGGAGTTGTCCAATTGCAGTT
CTTCTTCGCATAGAAATGCAATTGGAATATTTACAAATCATAATCAACAG
AGCCCCAGGGGCAAAATAGACATTCTATTTGTCAGCTGTTGCTGCTGAGC
CTGCTGGTGAACACCATTGCAAGCAGTTGCTGAGCCTGCTGGTGAACACC
ATTGCAAGCAGTTGCTGAGCCTGCTCGTGAACACCATTGCAAGCCATTGC
TGTCAAGCTTCATGAGTTAAATTTAACTGTTAGAGAGGAGAAAAGCATAG
CAGTCATTTGGAACGATCTCGTGGGGTGGGACTAAGCCATAATGATGTGT
CTGGCTCAAGCAGCAATGCTGTCCCTGCTCAGAACTGTGGTCTCTGTGGA

FIGURE 5

GAACCTAGTACAGAGCCTGAGCAAGGCAATGGACCACCTCCTTTTCCTCA
AACTGATGGATCACCACTGTTAATCTATACCAGGTGTGAAAAAGGGCATT
TCCCAAAACATACACAAAACACAACTTACTCTATTTCCCCTCCATTTCAG
CAAATGTGTTCTTGGTTCATGGCACTGGCACCCAGCGCATGGGCACCAGG
TAGGTGTTCCTCGAGTGCCAACGGCGGGGCCTGCCACCCCTGCTTCCTTT
CTGGGCAAGCTCCCATTTTAACTTTTCCCTGTTAAAAATTGTACTGATGT
TTTAAACTCGTAACTATACCACCCTTCTCACTGGTGTATTTTGAAAACCA
CCACAGTCCGATGTCCTGTGCCATGTCTGTCATTCCTGTTGAACAGACTC
TTCCTGTCTGGTGACCACCTATGCTTCAGTGCATTTTCTAAACAATTTCT
CAAACTGATCTGCTCCCGGGTGCTTTCTTACCACCATCTCTGGTGACTTA
GCCTCAAATTGGTGTTTTTCTTTCCCAAGAATTCATCTTAAGGGTTAGCT
TTGAGTATGTTACTGTCCTTCTATGACAAGACCAAAGGAACAAACTCCTT
GGTGAATCTGGAGTTCAGCTTTAGTTTTCAAACACAATCTGAAATCTGTT
AGTAAAATGAATTCTGGGTAAAAATGAGACTCAAGAGGCATCACTGGCCA
TTGTACCAGATTATTCCTGCTCGCCAGTGAGAGGCGTTTAAGTCGCGCTG
TGCATCGTACCTTCAGTTTAGGCTGTTTTCATGACCTCACATCTGTGTAG
CACAAAGGGTGTTATCTTCCTATAGCAGATCAACAGTGGCAGCTCACAGT
ACCAGCCAGCTTTAGACCTCCCAGTGCAAAGCCCAGCGCAGCACTAAGCC
TTAAGCCACCCAAGGGCTGACACACCATGCAGAGCTACGAAATCTGTGCT
GAGAAAATGCCATCTGGAAAGATTTTTAACCCATTCATTCTTAGTGTGAA
TTTTCTAAAATAGAAGTATAAAAAAAAAGTCTCTATAATCTGATAGGAAA
CCGTCTTTTCCCAAGTGAATTCCCTTTTATTCATGAGAACAAAAGTACTT
AGATACTATAAAACCTTAATTTCCCCAACTTTTAAAATAAAATGGAATAT
TTGCTGAATGAATTAAGCATAGAAAATGTCAACCCATCAGAATTATATTT
ATACATATTGGTGGATAAGTTCTTGTCTTCCCACATCAGAGAATTTTTTT
TTAAACCAAAATGGGCAGAATAGCATTTTGTATGTTAGAGACCATTCTTT
TCTAGTCCTTCCAAACATTCCTGTGTCACTTAAGAATGTTAGTAGAGGTC
GCTTCTTTGGAAATTGAGGACTTTTTTTTTTCCTTTGAAAGTATGGCATC
TGAAGAACACAGTATCATGACTCGGGTGGAAAGGGAGGCAGAAGCCATTT
TTCTTTTAGATGAAAAGCATTCTGTGTGATTGTTGTATAATAAATTGATT
TTTACACTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

FIGURE 5 (Cont'd)

>proteinssc2, 292 aa
MEQLKAFDNEVNAFLDNMFGPRDSRVRGWFLLDSYLPTFILTITYLLSIW
LGNKYMKNRPALSLRGILTLYNLAITLLSAYMLVELILSSWEGGYNLQCQ
NLDSAGEGDVRVAKVLWWYYFSKLVEFLDTIFFVLRKKTSQITFLHVYHH
ASMFNIWWCVLNWIPCGQSFFGPTLNSFIHILMYSYYGLSVFPSMHKYLW
WKKYLTQAQLVQFVLTITHTLSAVVKPCGFPFGCLIFQSSYMMTLVILFL
NFYIQTYRKKPVKKELQEKEVKNGFPKAHLIVANGMTDKKAQ*

FIGURE 6

```
       1                                                                    65
SSC1                                                      MEA VVNLYHELMK HADPR
SSC2                                               MEQLKAFDNE VNAPLDNMFG PRDSR
CIG30                                                MDTSMNF SRGLKMDLMQ PYDFE
SUR4   MNTTTSTVIA AVADQFQSLN SSSSCFLKVH VPSIENP.FG IELWPIFSKV FEYFSG..YP AEQFE
FEN1           MN SLVTQYAAPL FERYPQLHDY LPTLERPFFN ISLWEHFDDV VTRVTNGRFV PSEFQ
ELO1               MVSDWKNFC LEKASR...F RPTIDRPFFN IYLWDYFNRA VGWATAGRFQ PKDFE

       66                                                                  130
SSC1   I..QSYPLMG SPLLITSILL TYVYFILSLG PRIMANRKPF QLRGFMIVYN FSLVILSLYI VYEFL
SSC2   V..RGWFLLD SYLPTPILTI TYLLSIW.LG NKYMKNRPAL SLRGILTLYN LAITLLSAYM LVELI
CIG30  TFQDLRPFLE EYWVSSFLIV VVYLLLIVVG QTYMRTRKSF SLQRPLILWS FFLAIFSILG TLRMW
SUR4   FIHNKTFLAN GYHAVSIIIV YYIIIFGGQA ILRALNASPL KFKLLFEIHN LFLTSISLVL WLLML
FEN1   FIAGELPLST LPPVLYAITA YYVIIFGGRF LLS..KSKPF KLNGLFQLHN LVLTSLSLTL LLLMV
ELO1   FTVGKQPLSE PRPVLLFIAM YYVVIFGGRS LVK..SCKPL KLRFISQVHN LMLTSVSFLW LILMV

       131                                                                 195
SSC1   MSGWLSTYTW RC.DPIDFSN SPEALRMVRV AWLFMLSKVI ELMDTVIFIL RKKDGQVTFL HVFHH
SSC2   LSSWEGGYNL QC.QNLDSAG EGD.VRVAKV LWWYYFSKLV EFLDTIFFVL RKKTNQITFL HVYHH
CIG30  KFMATVMFTV GLKQTVCFAI YTDDAVVRFW SPLFLLSKVV ELGDTAFIIL RKR..PLIFV HWYHH
SUR4   EQLVPMVYHN GLFWSIC.SK EAFAPKLVTL YYLNYLTKFV ELIDTVFLVL RRK..KLLFL HTYHH
FEN1   EQLVPIIVQH GLYFAIC.NI GAWTQPLVTL YYMNYIVKFI EFIDTFFLVL KHK..KLTFL HTYHH
ELO1   EQMLPIVYRH GLYFAVC.NV ESWTQPMETL YYLNYMTKFV EFADTVLMVL KHR..KLTFL HTYHH

       196                                                                 260
SSC1   SVLPWSWWWG IKIAPGGMGS FHAMINSSVH VVMYLYYGLS ALGPVAQPYL WWKKHMTAIQ LIQFV
SSC2   ASMFNIWWCV LNWIPCGQSF FGPTLNSFIH ILMYSYYGLS VF.PSMHKYL WWKKYLTQAQ LVQFV
CIG30  STVLLFTSFG YKNKVPSGGW F.MTMNFGVH SVMYTYYTMK A.AKLKHPNL .LPMVITSLQ ILQMV
SUR4   GATALLCYTQ LIGRT.SVEW VVILLNLGVH VIMYWYYFLS SCG....IRV WWKQWVTRFQ IIQFL
FEN1   GATALLCYTQ LMGTT.SISW VPISLNLGVH VVMYWYYFLA ARG....IRV WWKEWVTRFQ IIQFV
ELO1   GATALLCYNQ LVGYT.AVTW VPVTLNLAVH VLMYWYYFLS ASG....IRV WWKAWVTRLQ IVQFM

       261                                                                 325
SSC1   LVSLHI.... ........SQ YYFMPSCNYQ YPIIIHLIWM YGT..IFFIL FSNFWYHSYT K...G
SSC2   LTITHT.... ........LS AVVKP.CGFP FGCLIFQS.S YMM..TLVIL FLNFYIQTYR KKPVK
CIG30  LGTIFG.... ........IL NYIWRQEKGC HTTTEHFFWS FMLYGTYFIL FAHFFHRAYL RPKGK
SUR4   IDLVFVYFAT YTFYAHKYL. DGILPNKGTC YGTQAAAAYG YLILTSYLLL FISFYIQSYK KGGKK
FEN1   LDIGFIYFAV YQKAVHLYF. .PILPHCGDC VGSTTATFAG CAIISSYLVL FISFYINVYK RKGTK
ELO1   LDLIVVYYVL YQKIVAAYFK NACTPQCEDC LGSMTAIAAG AAILTSYLFL FISFYIEVYK RGSAS

       326
SSC1   KRLPRAVQQN GAPATTKVKA N
SSC2   KELQEKEVKN GFPKAHLIVA NGMTDKKAQ
CIG30  VASKSQ
SUR4   TVKKESEVSG SVASGSSTGV KTSNTKVSSR KA
FEN1   TSRVVKRAHG GVAAKVNEYV NVDLKNVPTP SPSPKPQHRR KR
ELO1   GKKKINKNN
```

FIGURE 7

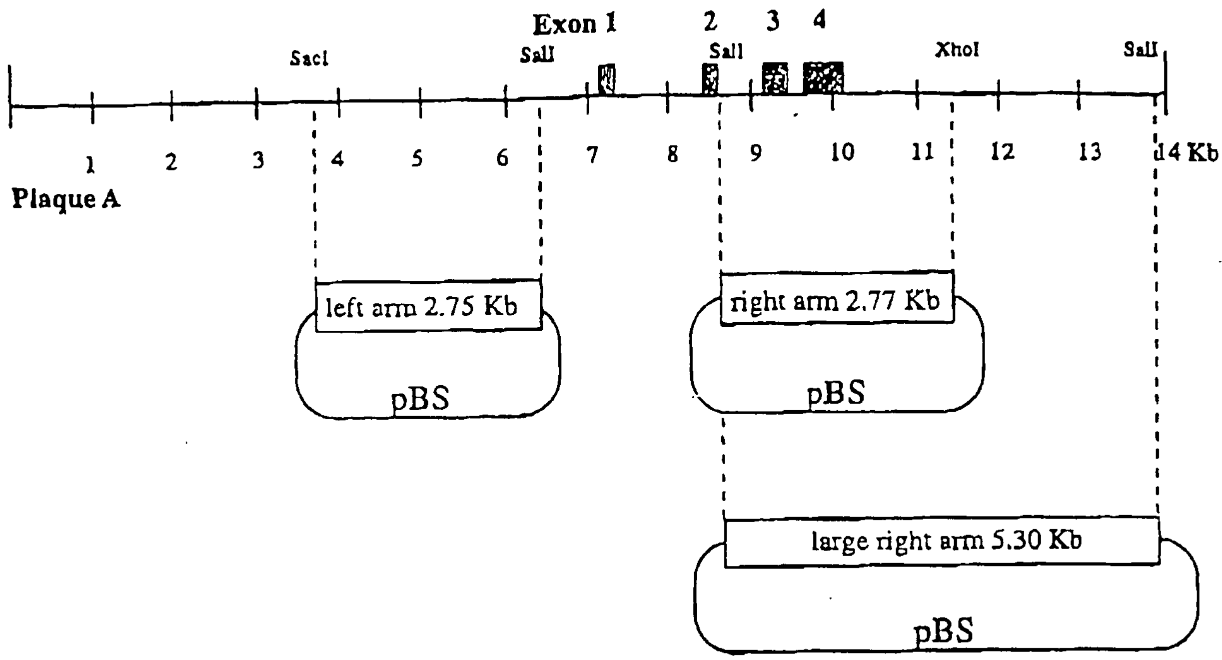
Exon 1
2
3
4
SacI
SalI
SalI
XhoI
SalI
1
2
3
4
5
6
7
8
9
10
11
12
13
14 Kb
Plaque A
left arm 2.75 Kb
pBS
right arm 2.77 Kb
pBS
large right arm 5.30 Kb
pBS

FIGURE 8

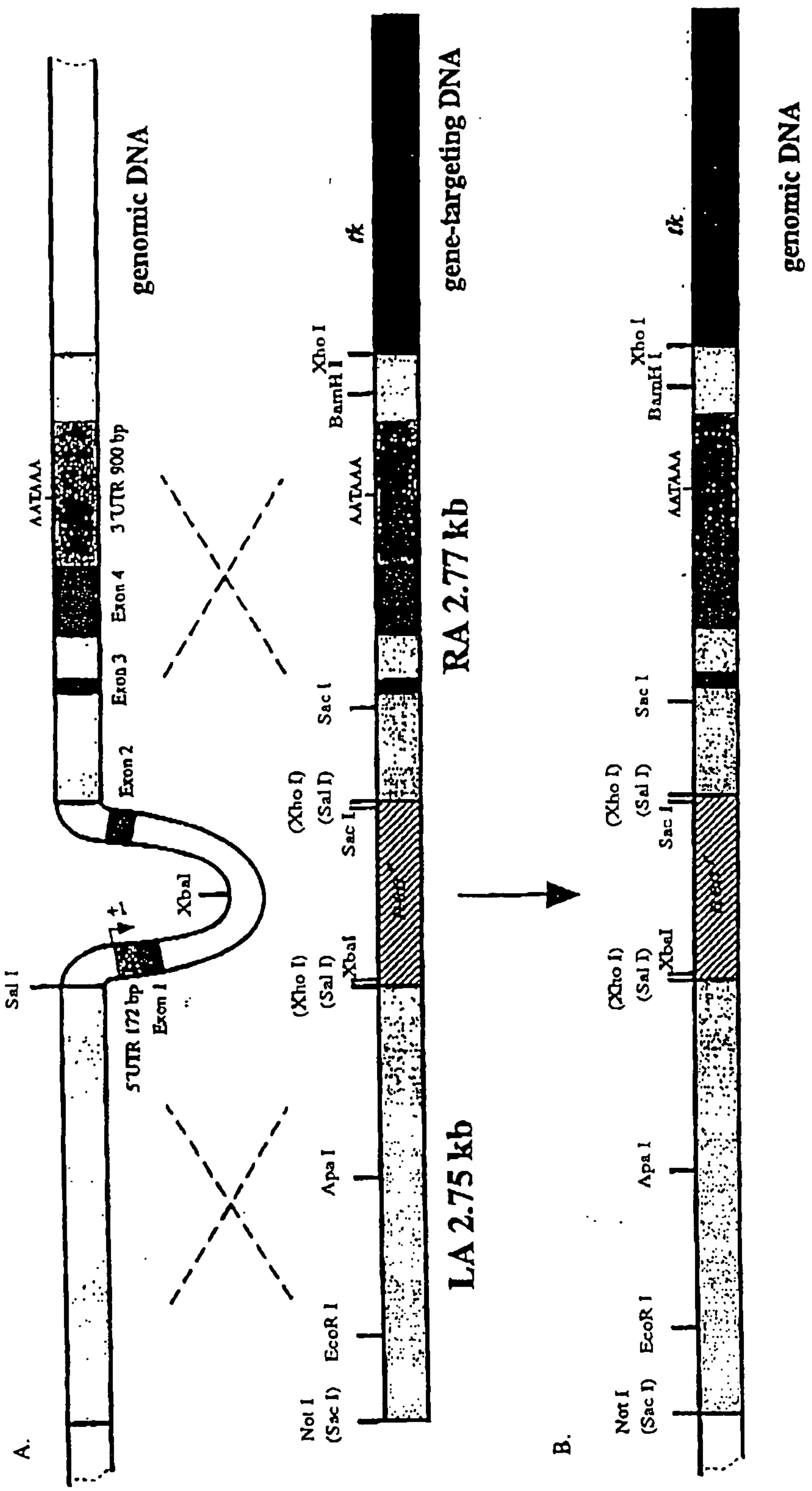
A.
genomic DNA
AATAAA
3'UTR 900 bp
Exon 4
Exon 3
Exon 2
XbaI
Sal I
5'UTR 172 bp
Exon 1
gene-targeting DNA
tk
Xho I
BamH I
AATAAA
RA 2.77 kb
Sac I
(Xho I)
(Sal I)
Sac I
XbaI
(Xho I)
(Sal I)
LA 2.75 kb
Apa I
EcoR I
Not I
(Sac I)
B.
genomic DNA
tk
Xho I
BamH I
AATAAA
Sac I
(Xho I)
(Sal I)
Sac I
XbaI
(Xho I)
(Sal I)
Apa I
EcoR I
Not I
(Sac I)

FIGURE 9

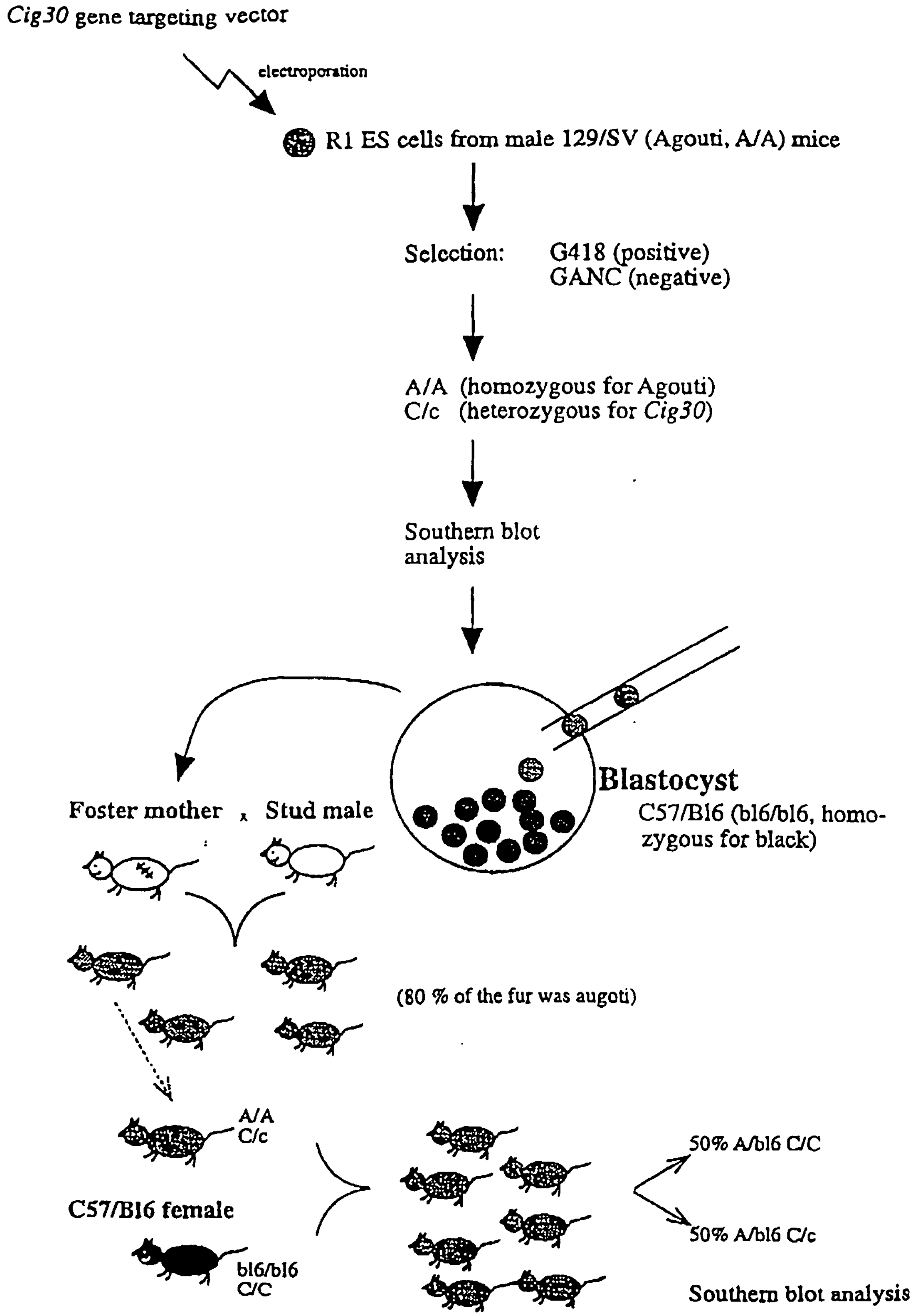
Cig30 gene targeting vector
electroporation
R1 ES cells from male 129/SV (Agouti, A/A) mice
Selection: G418 (positive)
GANC (negative)
A/A (homozygous for Agouti)
C/c (heterozygous for Cig30)
Southern blot
analysis
Blastocyst
C57/B16 (bl6/bl6, homozygous for black)
Foster mother x Stud male
(80 % of the fur was augoti)
A/A
C/c
C57/B16 female
bl6/bl6
C/C
50% A/bl6 C/C
50% A/bl6 C/c
Southern blot analysis

FIGURE 10